JOURNAL OF EXPERIMENTAL BOTANY

AN OFFICIAL ORGAN OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY

Volume 11 *September* 1960 *Number* 33

CONTENTS

D. D. Davies: The Purification and Properties of Glycolaldehyde Dehydrogenase 289

A. P. Wilkinson and D. D. Davies: Some Enzymic Steps in the Metabolism of Serine and Glycine by Higher Plants. I. 296

L. Fowden: The Metabolism of Labelled Lysine and Pipecolic Acid by *Acacia* Phyllodes 302

A. E. Flood, A. C. Hulme, and L. S. C. Wooltorton: The Organic Acid Metabolism of Cox's Orange Pippin Apples. I. 316

Tsin-tze Chan and I. F. Bird: Starch Dissolution in Tobacco Leaves in the Light 335

A. N. Lahiri and L. J Audus: Growth Substances in the Roots of *Vicia faba* 341

J. E. Gander: Growth Response of *Triticum* Coleoptiles to Non-dialysable Milk Fractions 351

N. Sunderland: The Production of the *Striga* and *Orobanche* Germination Stimulants by Maize Roots. II. 356

E. J. Hewitt and P. Tatham: Interaction of Mineral Deficiency and Nitrogen Source on Acid Phosphatase Activity in Leaf Extracts 367

K. Blaim: The Effect of Coumarin on Uptake of Water by Seeds . . . 377

C. E. L. Hepton and R. D. Preston: Electron Microscopic Observations of the Structure of Sieve-connexions in the Phloem of Angiosperms and Gymnosperms 381

C. North: Studies in Morphogenesis of *Brassica oleracea* L. II 395

Elizabeth M. C. Turner: The Nature of the Resistance of Oats to the Take-all Fungus 403

A. D. McKelvie: Cherelle Wilt of Cacao. II 413

OXFORD: AT THE CLARENDON PRESS

JOURNAL OF
EXPERIMENTAL BOTANY

Editorial Committee: W. T. WILLIAMS (Editor), E. ASHBY, G. E. BLACKMAN, R. BROWN, F. G. GREGORY, W. O. JAMES, W. H. PEARSALL, R. D. PRESTON, M. THOMAS.

THE JOURNAL OF EXPERIMENTAL BOTANY is sponsored by the Society for Experimental Biology as a medium for the publication of papers in the field of plant physiology, biochemistry, biophysics, experimental agronomy, and related topics.

Three numbers comprise a volume and they appear at four-monthly intervals. Subscription price 55*s*. per volume. Single numbers 22*s*. net.

NOTICE TO CONTRIBUTORS

Papers for publication should be addressed to Professor W. T. Williams. The right of publication in this journal is not restricted to members of the Society and contributions from research workers both in Great Britain and abroad will be welcomed.

Manuscripts must be typewritten with double spacing. Standard botanical and chemical terminology and abbreviations should be used and contributors are asked to cite references in standard form and present the summary at the beginning rather than at the end of their papers.

Contributors will receive one proof in page. An allowance of 10*s*. per sheet of sixteen pages is made for alterations in the proof, printers' errors excepted, and contributors will be responsible for any excess.

Contributors receive twenty-five copies of offprints free of charge, and may purchase further copies at rates which will be quoted on application to the publisher.

ILLUSTRATIONS

All figures, both text-figures and plates, must be numbered in a single series (figs. 1, 2, 3, &c.). There must not be one numbered series of text-figures and another separately numbered series of plates.

No figure should be sent that will not fit easily into an envelope 40 × 30 cm (about 16 × 12 inches).

The lettering of all figures should be clearly written on tracing paper bearing accurate outlines of the objects or graphs indicated by the lettering.

A typewritten (double-spaced) list of the legends of all figures must be provided. Each legend must be complete in itself. Remarks applying to more than one figure will not be printed.

Text figures and graphs. These must be drawn in black process ink with graphs preferably on quarto size mm graph paper.

Plates. When several photomicrographs are grouped to form a single plate, they should, if possible, be arranged to occupy an area of approximately the right shape to fill the available space (19 × 12 cm), and they must be neatly pasted to a single piece of white cardboard. The photomicrographs in a single plate must be labelled A, B, C, &c. Room should be left to include the legend below the photomicrographs on the plate rather than in a separate list of plates.

[*continued on p. iii of cover*

The Purification and Properties of Glycolaldehyde Dehydrogenase

D. D. DAVIES

Botany Department, King's College, London

Received 24 December 1959

SUMMARY

Evidence is presented that glycolaldehyde is a substrate for an aldehyde dehydrogenase present in pea seedling mitochondria. The enzyme has been partially purified and its properties are reported. The relevance of the reaction to the metabolism of the cell is discussed.

INTRODUCTION

GLYCOLALDEHYDE is a postulated intermediate in the biosynthesis of glycine (Weissbach and Horecker, 1955; Davies, Hanford, and Wilkinson, 1959):

'X' → glycolaldehyde → glycolate → glyoxalate → glycine

The steps between glycolate and glycine have been studied with preparations from higher plants (Clagett, Tolbert, and Burris 1949; Kenten and Mann, 1952; Zelitch and Ochoa, 1953; Wilson, King, and Burris, 1954) but the formation of glycolate from glycolaldehyde has not been previously reported.

METHODS

Chemicals. Glycolaldehyde-2-^{14}C was prepared by heating DL-serine-3-^{14}C (1·8 mg. 0·1 mCuries), ninhydrin (15 mg.) and formic acid (1 ml., 1·0 N) in a water bath at 80° C. On shaking the tube containing the reactants a blue colour appeared which quickly faded. Heating was continued until the blue colour no longer appeared on shaking. The tube was cooled, water (3 ml.) was added, and the solution passed through a small column of granulated charcoal. Immediately radioactivity was detected emerging from the column the solution was collected and the column washed with 2 ml. of water. The solution was shaken with platinum black (20 mg.) and after an hour with the monobed resin Amberlite M.B-3, which was added in small amounts until the colour of the resin was persistent. The resin was removed by filtration and, after washing, the volume of the solution was adjusted to 10 ml. Analysis by the method of Dickens and Williamson (1958) indicated a 90 per cent. yield of glycolaldehyde. Chromatography in ethanol/ammonia/water (80 : 10 : 10), propanol/ammonia (84 : 16), and 80 per cent. pyridine, showed the presence of a single radioactive compound with the Rf of authentic

JOURNAL OF
EXPERIMENTAL BOTANY

Editorial Committee: W. T. WILLIAMS (Editor), E. ASHBY, G. E. BLACKMAN, R. BROWN, F. G. GREGORY, W. O. JAMES, W. H. PEARSALL, R. D. PRESTON, M. THOMAS.

THE JOURNAL OF EXPERIMENTAL BOTANY is sponsored by the Society for Experimental Biology as a medium for the publication of papers in the field of plant physiology, biochemistry, biophysics, experimental agronomy, and related topics.

Three numbers comprise a volume and they appear at four-monthly intervals. Subscription price 55*s*. per volume. Single numbers 22*s*. net.

NOTICE TO CONTRIBUTORS

Papers for publication should be addressed to Professor W. T. Williams. The right of publication in this journal is not restricted to members of the Society and contributions from research workers both in Great Britain and abroad will be welcomed.

Manuscripts must be typewritten with double spacing. Standard botanical and chemical terminology and abbreviations should be used and contributors are asked to cite references in standard form and present the summary at the beginning rather than at the end of their papers.

Contributors will receive one proof in page. An allowance of 10*s*. per sheet of sixteen pages is made for alterations in the proof, printers' errors excepted, and contributors will be responsible for any excess.

Contributors receive twenty-five copies of offprints free of charge, and may purchase further copies at rates which will be quoted on application to the publisher.

ILLUSTRATIONS

All figures, both text-figures and plates, must be numbered in a single series (figs. 1, 2, 3, &c.). There must not be one numbered series of text-figures and another separately numbered series of plates.

No figure should be sent that will not fit easily into an envelope 40 × 30 cm (about 16 × 12 inches).

The lettering of all figures should be clearly written on tracing paper bearing accurate outlines of the objects or graphs indicated by the lettering.

A typewritten (double-spaced) list of the legends of all figures must be provided. Each legend must be complete in itself. Remarks applying to more than one figure will not be printed.

Text figures and graphs. These must be drawn in black process ink with graphs preferably on quarto size mm graph paper.

Plates. When several photomicrographs are grouped to form a single plate, they should, if possible, be arranged to occupy an area of approximately the right shape to fill the available space (19 × 12 cm), and they must be neatly pasted to a single piece of white cardboard. The photomicrographs in a single plate must be labelled A, B, C, &c. Room should be left to include the legend below the photomicrographs on the plate rather than in a separate list of plates.

[*continued on p. iii of cover*

The Purification and Properties of Glycolaldehyde Dehydrogenase

D. D. DAVIES

Botany Department, King's College, London

Received 24 December 1959

SUMMARY

Evidence is presented that glycolaldehyde is a substrate for an aldehyde dehydrogenase present in pea seedling mitochondria. The enzyme has been partially purified and its properties are reported. The relevance of the reaction to the metabolism of the cell is discussed.

INTRODUCTION

GLYCOLALDEHYDE is a postulated intermediate in the biosynthesis of glycine (Weissbach and Horecker, 1955; Davies, Hanford, and Wilkinson, 1959):

'X' → glycolaldehyde → glycolate → glyoxalate → glycine

The steps between glycolate and glycine have been studied with preparations from higher plants (Clagett, Tolbert, and Burris 1949; Kenten and Mann, 1952; Zelitch and Ochoa, 1953; Wilson, King, and Burris, 1954) but the formation of glycolate from glycolaldehyde has not been previously reported.

METHODS

Chemicals. Glycolaldehyde-2-^{14}C was prepared by heating DL-serine-3-^{14}C (1·8 mg. 0·1 mCuries), ninhydrin (15 mg.) and formic acid (1 ml., 1·0 N) in a water bath at 80° C. On shaking the tube containing the reactants a blue colour appeared which quickly faded. Heating was continued until the blue colour no longer appeared on shaking. The tube was cooled, water (3 ml.) was added, and the solution passed through a small column of granulated charcoal. Immediately radioactivity was detected emerging from the column the solution was collected and the column washed with 2 ml. of water. The solution was shaken with platinum black (20 mg.) and after an hour with the monobed resin Amberlite M.B-3, which was added in small amounts until the colour of the resin was persistent. The resin was removed by filtration and, after washing, the volume of the solution was adjusted to 10 ml. Analysis by the method of Dickens and Williamson (1958) indicated a 90 per cent. yield of glycolaldehyde. Chromatography in ethanol/ammonia/water (80 : 10 : 10), propanol/ammonia (84 : 16), and 80 per cent. pyridine, showed the presence of a single radioactive compound with the Rf of authentic

glycolaldehyde. The preparation was stable when stored at −15° C. for a month, but when subsequently examined after nine months the preparation was found to be contaminated with a radioactive compound which is possibly a tetrose formed by aldol condensation. Crystalline alcohol dehydrogenase was prepared from yeast according to Racker (1950). Glycolaldehyde-2-phosphate was kindly supplied by Dr. Z. Dische, all other chemicals were obtained commercially.

Analytical. Spectrophotometric measurements were made at 30° in a Unicam S.P. 500 spectrophotometer fitted with a constant temperature device. Protein was determined by the method of Warburg and Christian (1942).

Preparation of an extract of pea mitochondria. Mitochondria were isolated from etiolated epicotyls of 7–10-day-old pea seedlings (*Pisum sativum* var. 'Alaska') according to the method of Bonner and Millerd (1953). The washed mitochondria were suspended in phosphate buffer (pH 7·8 0·1 M.) containing cysteine (5 mg./100 ml.) and frozen by immersion in a freezing mixture of solid carbon dioxide and acetone. After thawing in a bath at 30° and freezing again, the suspension was stored overnight at −18°. The following day the preparation was placed in a bath at 30° and, immediately it had completely thawed, was transferred to an ice-water bath for 10 minutes before centrifuging at 30,000 *g* for 20 minutes to give a clear supernatant.

Enzyme assay. Glycolaldehyde dehydrogenase was measured at a wavelength of 340 mμ in silica cells of light path 1·0 cm. containing glycolaldehyde (2×10^{-4} M.), phosphate buffer (pH 7·4 0·1 M.) DPN (0·5 mg.). The reaction was started by the addition of enzyme previously incubated with cysteine. Under these conditions the activity is proportional to the enzyme concentration (Fig. 1). A unit of enzyme activity is defined as the amount of enzyme producing an optical density change at 340 mμ of 0·01 per minute. Specific activity is defined as the number of units per mg. protein.

RESULTS

1. *Demonstration of glycolaldehyde dehydrogenase*

Because of the reactivity of glycolaldehyde, particularly under alkaline conditions, it is important to have adequate controls to check non-enzymic reactions. In the present investigation, two non-enzymic reactions have been observed by spectrophotometry. Under alkaline conditions, glycolaldehyde gives rise to a compound or compounds with absorption maxima at 335 mμ and 240 mμ, but the rate of this reaction is a function of pH and is negligible at pH 7·8 (Fig. 2). Glycolaldehyde also reacts under alkaline conditions with diphosphopyridine nucleotide (DPN), presumably by attacking the C4 position of the nicotinamide ring (Burton and Kaplan, 1954) to form a compound with an absorption spectrum similar to that of reduced DPN (Fig. 3). Sincet he rate of this reaction was also negligible at pH 7·8, enzyme assays were carried out at this pH.

On the addition of glycolaldehyde (0·6 μMoles) to a cuvette containing mitochondrial extract (1 ml.), cysteine (100 μMoles), DPN (0·5 μMoles), and phosphate buffer (pH 7·8 0·1 M.) in a total volume of 3 ml., the optical density at 340 mμ increased with time. Evidence that the increased optical density was due to DPNH was obtained by the addition of crystalline alcohol dehydrogenase and acetaldehyde (20 μMoles), which produced a rapid fall in

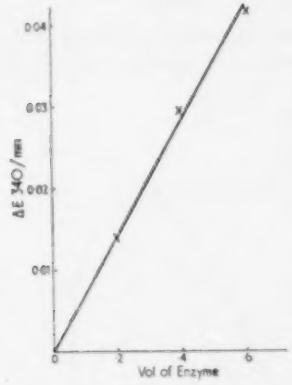


FIG. 1

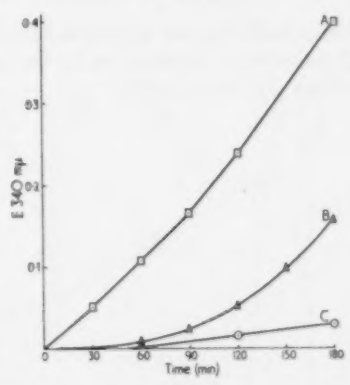


FIG. 2

FIG. 1. Effect of enzyme concentration on glycolaldehyde oxidation. Conditions were those of the standard assay described in the text.

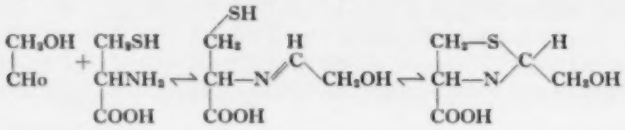
FIG. 2. Non-enzymic reactions involving DPN and glycolaldehyde. Optical densities measured at 340 mμ.

Curve A. DPN (0·34 μMoles), glycolaldehyde 1·8 mg., glycine buffer pH 9·5 0·066 M. in a total volume of 3 ml.
Curve B. DPN omitted.
Curve C. Conditions as for A except glycine buffer replaced by phosphate 0·066 buffer (M.) pH 7·8.

the optical density (Fig. 4), presumably due to the oxidation of reduced DPN.

Evidence for the formation of glycolate was obtained by using glycolaldehyde-2-^{14}C (0·6 μMoles 3·5 μCuries) in the standard assay system. When the optical density had reached a maximum the contents of the cuvette were transferred to a conical centrifuge tube and placed in a boiling-water bath for 5 minutes. After cooling, carrier sodium glycolate (0·2 mg.) was added and the coagulated protein removed by centrifugation. The supernatant was passed through a column of Amberlite IRA 400 (previously activated with sodium carbonate and washed until the pH of the effluent was below 7). The column was washed with water and glycolate was eluted with ammonium hydroxide (10 per cent.). Chromatography in propanol/ammonia and autoradiography showed the presence of two radioactive compounds in the eluate. The major radioactive compound was identified as glycolate by chromatography in a variety of solvents; the minor radioactive compound, which had less than 3 per cent. of the activity found in the glycolate area, has been

tentatively identified as 2-hydroxymethylthiazolidene-4-carboxylic acid, which may be formed non-enzymically by the following reaction



This compound does not appear to have been reported previously, but formation of thiazolidene derivatives by the reaction of cysteine and aldehydes is well known (Schubert, 1936).

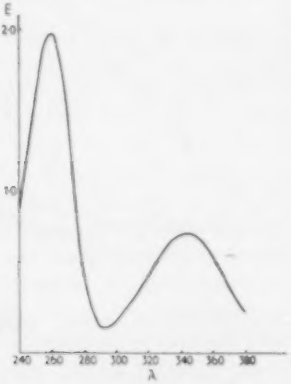


FIG. 3

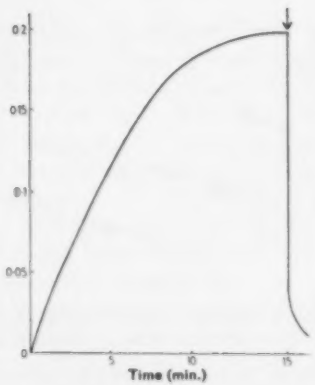


FIG. 4

FIG. 3. Absorption spectrum of compound formed from glycolaldehyde and DPN. Cell contained DPN (0·4 μMoles), glycolaldehyde (1·8 mg.) and glycine buffer (pH 10·0·066 M.) in a total volume. The spectrum was measured 5 hours after mixing and is corrected for absorption due to glycolaldehyde alone.

FIG. 4. Spectrophotometric evidence for the reduction of DPN by glycolaldehyde. Cell contents as in text. Acetaldehyde and alcohol dehydrogenase added at time indicated by arrow.

2. *Concentration and Partial Purification of Glycolaldehyde dehydrogenase*

The clear mitochondrial extract was brought to 50 per cent. saturation by the addition of powdered ammonium sulphate and after standing for 5 minutes the precipitate was collected by centrifuging at 10,000 *g* for 5 minutes. The precipitate was dissolved in phosphate buffer (30 ml. pH 7·4 0·2 M.) containing cysteine (15 mg.) and the solution fractionally precipitated with ammonium sulphate. The fraction precipitating between 30 and 40 per cent. saturation was used in all studies. The purification achieved is shown in Table I.

3. *Properties of the partially purified enzyme*

Effect of Cysteine. The purified enzyme had no activity in the absence of cysteine. The enzyme was incubated with varying concentrations of cysteine

TABLE I

Purification of Glycolaldehyde dehydrogenase

Fraction	Volume ml.	Total protein mg.	Total activity (units)	Specific activity	Recovery %	Purification
Extract	90	1,250	1,230	1	—	—
1st $(NH_4)SO_4$ precipitation	30	510	1,080	2	88	2
2nd $(NH_4)SO_4$ fractionation						
30–40	10	70	800	11·4	65	11
40–50	10	270	120	0·5	—	—

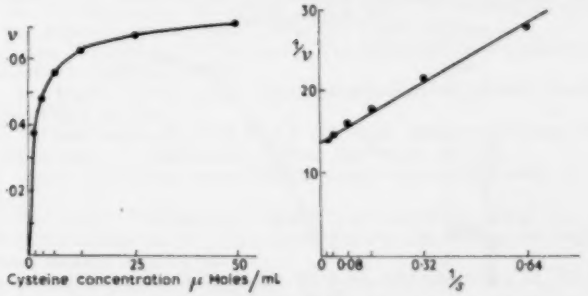


FIG. 5. Effect of cysteine concentration on the activity of glycolaldehyde dehydrogenase. Enzyme (specific activity 11) was incubated with cysteine at 0° for 15 minutes before being added to the substrate.

at 0° for 15 minutes before measuring the activity. The activity of the enzyme is related to the cysteine concentration by a Michaelis-Menten type curve in which half maximum velocity is attained when the cysteine concentration is 1·5 μMoles/ml. (Fig. 5). It should be noted that this concentration relates to the incubation at 0°, the concentration *during* the assay being one-third of this value.

Effect of glycolaldehyde concentration. At low substrate concentration the kinetics of glycolaldehyde dehydrogenase approximate to the Michaelis-Menten formulation, but inhibition is observed when the glycolaldehyde concentration is in excess of 2×10^{-4} M. (Fig. 6).

Effect of pH. The enzyme has only slight activity at pH 6·0 and an apparent optimum at pH 8·0. The effect of more alkaline conditions was not studied because of interference by non-enzymic reactions.

Specificity. The enzyme reacts with triphosphopyridine nucleotide (TPN) in the place of DPN and with acetaldehyde and glycolaldehyde-2-phosphate in the place of glycolaldehyde. With the three aldehydes at the same concentration (10^{-4} M.), the rates of reduction with glycolaldehyde acetaldehyde and phosphoglycolaldehyde were in the ratios 10 : 4 : 1.

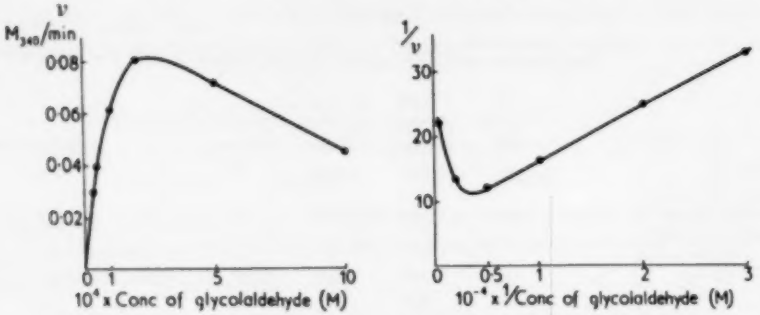


FIG. 6. Effect of glycolaldehyde concentration on the activity of glycolaldehyde dehydrogenase.

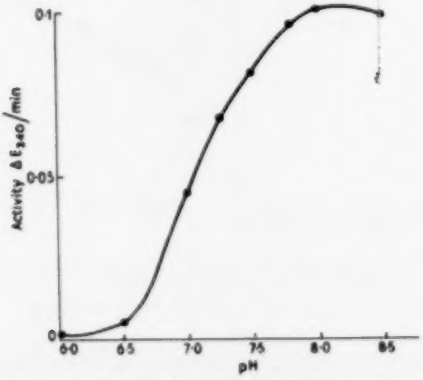


FIG. 7. Effect of pH on the activity of glycolaldehyde dehydrogenase. Phosphate buffer (0·1 M.) was used except at pH 8·5, when it was replaced by tris (hydroxymethyl)-aminomethane (0·1 M.).

DISCUSSION

The enzyme has a high affinity for glycolaldehyde (Km 7×10^{-5} M.). The inhibition of the enzyme by concentrations of glycolaldehyde in excess of 2×10^{-4} M. can be explained by the assumption that glycolaldehyde can combine with the enzyme at two sites, combination at the second site reducing the catalytic activity of the enzyme (Alberty, 1956). This explanation is in accordance with the high reactivity of glycolaldehyde (Hough and Jones, 1956).

The demonstration of a particular reaction does not establish the participation of that reaction in the metabolism of the cell. However, the dehydrogenase is present in leaves (Zelitch, 1958) and feeding experiments, to be reported elsewhere, have supported the view that glycolaldehyde is a precursor of glycine. When glycolaldehyde-2-^{14}C was fed to spinach leaves kept in

darkness, radioactivity was found mainly in glycine and serine. When supplied to washed disks of turnip, the label from glycolaldehyde-2-^{14}C was found to enter glycine, serine, glutathione, methylcysteine-sulphoxide, methionine, and a number of unidentified compounds. Further work is necessary to determine the quantitative significance of these reactions.

LITERATURE CITED

ALBERTY, R. A. (1956). Enzyme Kinetics. *Adv. Enz.* **17**, 1.

BONNER, J., and MILLERD, A. (1953). Oxidative phosphorylation by plant mitochondria. *Arch. Biochem.* **42**, 135.

BURTON, R. M., and KAPLAN, N. O. (1954). The reaction of pyridine nucleotides with carbonyl compounds. *J. Biol. Chem.* **206**, 283.

CLAGETT, C. O., TOLBERT, N. E., and BURRIS, R. H. (1949). Oxidation of α-hydroxyacids by enzymes from plants. Ibid., **178**, 977.

DAVIES, D. D., HANFORD, J., and WILKINSON, A. P. (1959). The metabolism of aspartic acid, serine and glycine in higher plants. *Symp. Soc. Explt. Biol.* **13**, 353.

DICKENS, F., and WILLIAMSON, D. H. (1958). The determination of hydroxypyruvate and glycolaldehyde. *Biochem. J.* **68**, 84.

HOUGH, L., and JONES, J. K. N. (1956). The biosynthesis of the monosaccharides. *Adv. in Carbohyd. Chem.* **11**, 185.

KENTEN, R. H., and MANN, P. J. G. (1952). Hydrogen peroxide formation in oxidations catalysed by plant and hydroxyacid oxidase. *Biochem. J.* **52**, 130.

RACKER, E. (1950). Crystalline alcohol dehydrogenase from baker's yeast. *J. Biol. Chem.* **184**, 313.

SCHUBERT, M. P. (1936). Compounds of thiolacids with aldehydes. Ibid., **114**, 341.

WARBURG, O., and CHRISTIAN, W. (1942). Isoliering und kristallisation des Garungferments Endolase. *Biochem. Z.* **310**, 384.

WEISSBACH, A., and HORECKER, B. L. (1955). In *Amino acid metabolism*. Ed. W. D. McElroy and B. Glass. Baltimore: Johns Hopkins Press.

WILSON, D. G., KING, K. W., and BURRIS, R. H. (1954). Transamination reactions in plants. *J. Biol. Chem.* **208**, 863.

ZELITCH, I., and OCHOA, S. (1953). Oxidation and reduction of glycolic and glyoxylic reductase from tobacco leaves. *J. Biol. Chem.* **201**, 707.

—— (1958). Private communication.

Some Enzymic Steps in the Metabolism of Serine and Glycine by Higher Plants

I. HYDROXYMETHYL TETRAHYDROFOLIC DEHYDROGENASE FROM TURNIPS

A. P. WILKINSON AND D. D. DAVIES

Botany Department, King's College, London

Received 10 February 1960

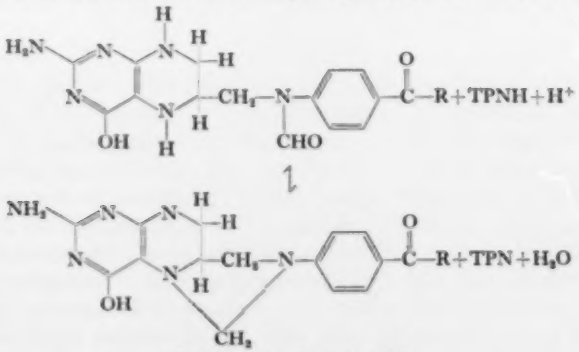
SUMMARY

The presence of the enzyme hydroxymethyl tetrahydrofolic dehydrogenase has been demonstrated in cell-free preparations from turnip hypocotyls. The enzyme is specific for triphosphopyridine nucleotide and, in conjunction with the enzyme serine aldolase, converts the hydroxymethyl group of serine into the formyl group of N^{10} formyl tetrahydrofolic acid. N^{10} formyl tetrahydrofolic acid formed enzymically from serine-3-^{14}C was converted to anhydroleucovorin and isolated by chromatography.

INTRODUCTION

FEEDING experiments have indicated that the carbon of formate can be transferred to the β position of serine (Tolbert and Cohan, 1953). The conversion of formate to the hydroxymethyl group of serine is essentially the reduction of a carboxyl group to the level of an aldehyde. On thermodynamic grounds such a reduction by a pyridine nucleotide linked enzyme is only possible if the carboxyl group is 'activated'. Experiments with animals and bacteria (Huennekens and Osborn, 1959) have shown that the active form of formate is N^{10} formyl tetrahydrofolic acid (formyl THFA). The results reported here establish the presence in turnips of a triphosphopyridine nucleotide (TPN) specific hydroxymethyl tetrahydrofolic dehydrogenase, which catalyses the reduction of N^{10} formyl THFA to hydroxymethyl THFA.

Formyl THFA + TPNH + H^+ ⇌ Hydroxymethyl THFA + TPN



(R represents glutamic acid)

MATERIALS AND METHODS

Chemicals. Tetrahydrofolic acid (THFA) was prepared by the catalytic reduction of folic acid. Folic acid (2·2 mg.) dissolved in an equivalent amount of sodium bicarbonate (0·5 ml.) was placed in the side arm of a Warburg flask and platinum black (10 mg.) and water (2·0 ml.) in the main compartment. The flask was gassed with hydrogen and shaken at 37° until the uptake of hydrogen stopped. Folic acid was then tipped and shaking continued until the theoretical hydrogen uptake was completed. The solution contains 2 μMoles DL THFA per ml.

Oxidized glutathione (GSSG) was prepared from the reduced form by oxidation with bromine. Formaldehyde was prepared from paraformaldehyde by heating paraformaldehyde with successive small volumes of water in a sealed tube at 100° until it had all dissolved. DL-Serine-3-^{14}C was obtained from the Radiochemical Centre, Amersham, and was found to be chromatographically pure.

Analytical. Spectrophotometric measurements were made in a Unicam S.P. 500 Spectrophotometer. The extinction coefficient of methenyl THFA (anhydroleucovorin) at pH 1·0 was taken as $2{\cdot}2 \times 10^7$ cm.^{2}/mol at 355mμ (Greenberg, Jaenicke, and Silverman 1955). Anhydroleucovorin was separated from serine and various products formed from THFA by chromatography on Whatman No. 1 paper, using 1 M. formic acid as the developing solvent.

RESULTS AND DISCUSSION

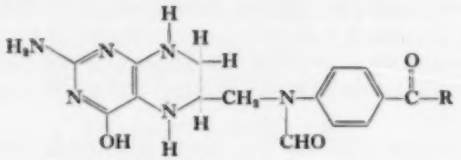
The enzyme extract was prepared by homogenizing turnip slices (100 gm.) in 2–3 volumes of cold potassium phosphate buffer (0·2 M. ph 7·4) in a bottom-drive blender. The suspension was strained through linen, and the cleared solution brought to 80 per cent. saturation with ammonium sulphate. After 15 minutes the protein was separated by centrifugation, taken up in the minimum volume of phosphate buffer and then dialysed for 2 hours against a running flow of phosphate buffer (pH 7·4 0·01 M.). The extract was cleared by centrifuging at 30,000$\times g$ for 10 minutes. All operations were carried out in a cold room at +2°.

Attempts to follow the reduction of TPN by hydroxymethyl THFA by means of direct spectrophotometry at 340 mμ, were only partially successful. Consequently the reaction was studied by converting formyl THFA to methenyl THFA (anhydroleucovorin) by treatment with acid and measuring the light absorption at 355 mμ.

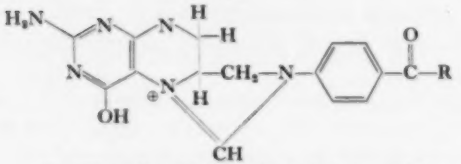
Active formaldehyde can be prepared by mixing an excess of formaldehyde with tetrahydrofolic acid. Such a preparation probably contains formaldehyde bound at the N^5, N^8, and N^{10} positions of THFA as well as the enzymically active form, which is probably the N^5, N^{10} bridge compound methylene THFA (Huennekens and Osborn, 1959).

The turnip enzyme (2 ml.) was incubated at 37° C. with formaldehyde (4 μMoles) DL-tetrahydrofolic acid (4 μMoles), TPN (2 mg.), $MgSO_4$

(2 μMoles), pyridoxal phosphate (0·1μ Moles), glutathione (10μ Moles) and phosphate buffer pH 7·4 (0·05 M.) in a total volume of 5 ml. and maintained anaerobic by layering with petroleum ether. At intervals, 0·5 ml. aliquots were removed and added to 0·5 ml. of trichloroacetic acid (10 per cent.), to



N^{10} formyl THFA



N^5, N^{10}-methenyl THFA (anhydroleucovorin)

precipitate protein and convert N^{10} formyl THFA to anhydroleucovorin. The mixture was centrifuged, 0·5 ml. of the supernatant was added to 2·5 ml. of water in a silica cell, and the optical density between 320 and 380 mμ measured against a zero time control.

Although the optical density at 355 mμ increased with the time of incubation, the observed spectrum was not characteristic of anhydroleucovorin. A shoulder at 350 mμ suggests the presence of some anhydroleucovorin but material absorbing light at 330 mμ interferes with the assay (Fig. 1).

Previous experiments (Wilkinson and Davies, 1958) have shown that turnip preparations similar to those employed in the present investigation contain the enzyme serine aldolase, which catalyses the reaction

$$\text{Serine} + \text{THFA} \rightleftharpoons \text{glycine} + \text{hydroxymethyl (THFA)}$$

Hydroxymethyl THFA produced by this reaction would presumably not produce the mixture of compounds formed by adding formaldehyde to THFA; consequently serine (40μ Moles) was used in place of formaldehyde in the assay system. The spectrum of the reaction product was then found to be similar to that of anhydroleucovorin (Fig. 1).

Under the conditions of the assay procedure, the amount of formyl THFA increased for the first 60 minutes of incubation but thereafter declined (Fig. 2A). The rate of formation of formyl THFA measured as the amount of formyl THFA produced in 60 minutes was found to be linearly related to the concentration of enzyme, provided the rate of formyl THFA production did not exceed 0·1 μMoles per ml. per hour (Fig. 2B).

The requirements for the two reactions involved in the formation of the

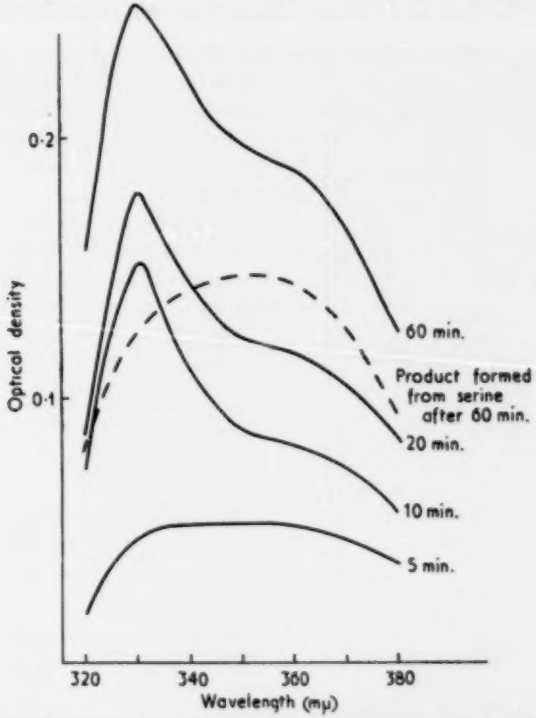


FIG. 1. Absorption Spectrum of acidified reaction products formed after incubating formaldehyde (4μ Moles), DL THFA (4μ Moles), TPN (2 mg.), $MgSO_4$ (2μ Moles), pyridoxal phosphate (0·1μ Moles), oxidized glutathione (10μ Moles), phosphate buffer pH 7·4 (0·05 M.), and turnip enzyme (2 ml.) in a total volume of 5 ml. for the times indicated. For comparison, the spectrum of the acidified products formed when serine (40μ Moles) replaced formaldehyde is included.

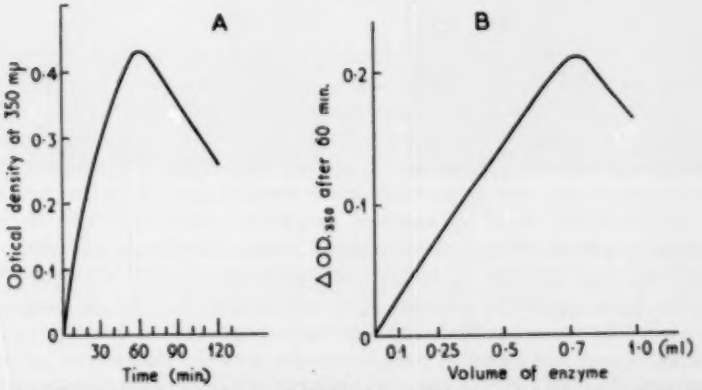


FIG. 2A. Rate of formation of formyl THFA (measured as anhydroleucovorin). Assay conditions as in Fig. 1 except that formaldehyde was replaced by serine (40μ Moles).
FIG. 2B. Effect of enzyme concentration on the rate of formation of formyl THFA.

formyl group of formyl THFA from the hydroxymethyl group of serine were studied by omitting components of the assay system, and the results shown in Table I are examples of the variability found in more than a dozen experiments. A number of factors may be required, but the results clearly established the enzymic nature of the reaction, the requirement for THFA and for TPN which cannot be replaced by diphosphopyridine nucleotide (DPN). The large blank reaction found in the absence of serine is associated with the proteolytic activity of the enzyme preparation. Immediately after dialysis amino-acids could not be detected in the enzyme preparation, but after incubation in the absence of substrates, amino-acids were detected by chromatography. It

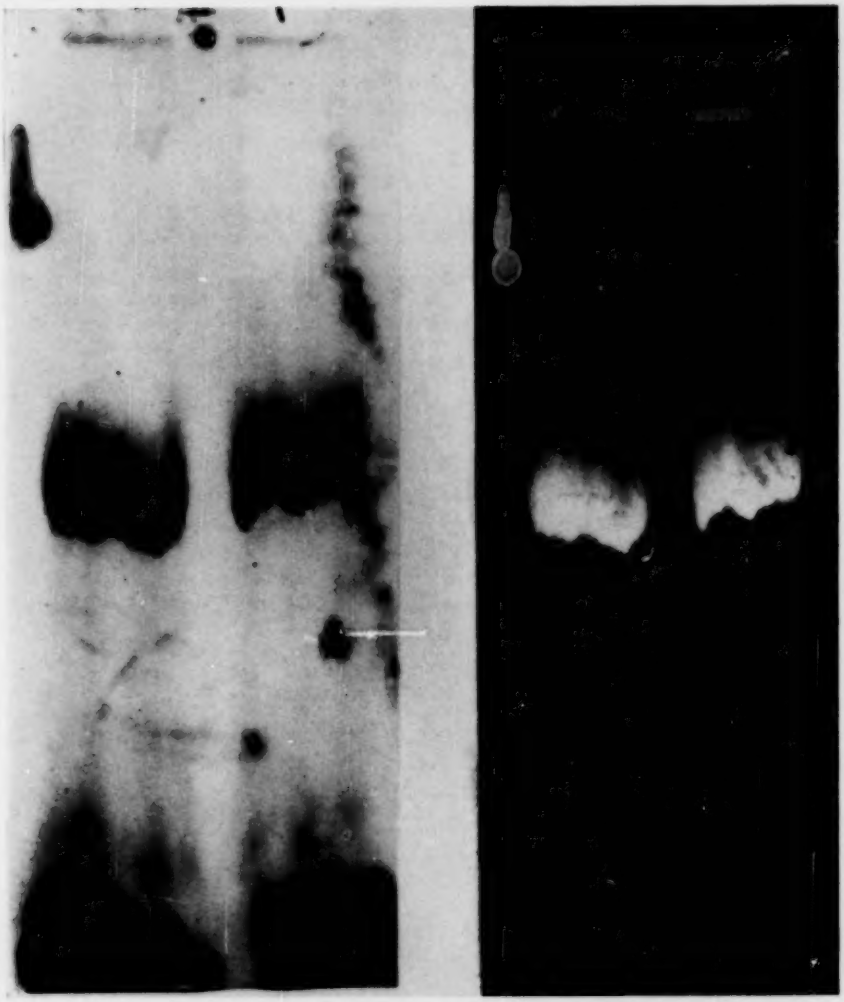
TABLE I

Requirements for the formation of formyl THFA from serine and THFA. The complete system contained L*-serine (10 μMoles), DL THFA (1 μMole), TPN (1 mg.), $MgSO_4$ (1 μMole), pyridoxal phosphate (0·1 μMole), oxidized glutathione (5 μMoles), phosphate buffer pH 7·4 (0·05 M.) and turnip enzyme (1·0 ml. in a total volume of 2·5 ml. The yield of formyl THFA measured as anhydroleucovorin is expressed as the increase in optical density at 350 mμ after an incubation of 60 minutes*

Conditions	Yield of formyl THFA			
	Expt. 1	Expt. 2	Expt. 3	Expt. 4
Complete system	2·42	2·78	2·64	2·6
No TPN	0·68	—	1·09	0·54
No TPN+DPN (1 mg.)	0·85	—	—	—
No Mg^{++}	2·1	—	—	—
No serine	2·2	2·4	1·8	1·6
No glutathione	2·26	—	—	—
No pyridoxal phosphate	2·22	—	—	—
No THFA	—	0	—	0
Boiled enzyme	—	0·2	—	0·3

was, however, necessary to obtain further evidence that the β-carbon of serine can become the formyl carbon of formyl THFA.

DL-serine-3-^{14}C was used in the assay system and after 60 minutes the reaction was stopped with trichloroacetic acid. The spectrum of the acidified reaction products was found to be similar to that of anhydroleucovorin. Anhydroleucovorin was separated from the reaction mixture by first removing the trichloroacetic acid by repeated extraction with ether and then by chromatography on Whatman No. 1 paper using molar formic acid. Anhydroleucovorin was detected by its intense fluorescence under ultraviolet light and the U.V. irradiated chromatogram photographed. The chromatogram was placed in contact with X-ray film for 1 week and on development the radioactive area was found to coincide exactly with the fluorescent anhydroleucovorin area (Fig. 3), thereby confirming the transfer of the hydroxymethyl group to THFA and its oxidation to formyl THFA.



Autoradiograph

Photograph under ultraviolet light

FIG. 3. Chromatographic evidence for the transfer of the β-carbon of serine to the formyl carbon of formyl THFA. On treatment with acid formyl THFA was converted to anhydroleucovorin which was isolated by chromatography in 1 M. formic acid. Serine-3-14 C ran at the solvent front. Anhydroleucovorin was detected by its fluorescence under ultraviolet light and by autoradiography

A. P. WILKINSON *and* D. D. DAVIES—PLATE I

ACKNOWLEDGEMENTS

We are indebted to Professor T. A. Bennet-Clark, F.R.S., for his interest and advice and to the Royal Society and the Agricultural Research Council for grants towards apparatus and chemicals.

LITERATURE CITED

GREENBERG, G. R., JAENICKE, L., and SILVERMAN, M. (1955). On the occurrence of N^{10} formyl THFA by enzymic formylation of THFA and on the mechanism of this reaction. *Biochim. Biophys. Acta* **17**, 589.

HUENNEKENS, F. M., and OSBORN, M. J. (1959). Folic acid coenzymes and one-carbon metabolism. *Adv. Enz.* **21**, 369.

TOLBERT, N. E., and COHAN, M. S. (1953). Products formed from glycolate in plants. *J. Biol. Chem.* **204**, 639.

WILKINSON, A. P., and DAVIES, D. D. (1958). Serine-glycine inter-conversion in plant tissues. *Nature*, London, **181**, 1070.

ADDENDUM

This investigation establishes only that the product of the *assay* is N^5N^{10}-methenyltetrahydrofolic acid. The immediate product of the dehydrogenase could be N^{10}-formyl- or N^5N^{10}-methenyltetrahydrofolic acid, which are interconvertible by the enzyme cyclohydrolase. (Osborn, M. J., and Huennekens, F. M. (1957). *Biochim. Biophys. Acta* **26**, 646.)

The Metabolism of Labelled Lysine and Pipecolic Acid by *Acacia* Phyllodes

L. FOWDEN

Botany Department, University College, London, W.C.1

Received 12 January 1960

SUMMARY

C^{14}-lysine and C^{14}- and H^3-pipecolic acids have been used to study the metabolism of the lysine family of amino-acids in *Acacia*, which contains 4-hydroxypipecolic acid as a characteristic component of the soluble-nitrogen fraction. Degradation of C^{14}-lysine was rapid and was far more extensive than that observed earlier in higher plants. Pipecolic acid was the major radioactive product in short-term experiments. After longer metabolic periods, radioactivity was distributed over a wide range of amino-acids, organic acids, and sugars. A tentative metabolic scheme is produced to explain these observations concerning lysine degradation. The distribution of radioactivity in the amino-acids of the protein present in the phyllodes was determined 24 hours after supplying C^{14}-lysine. Specific activities of free and bound amino-acids are compared at this time. Hydroxyproline forms a notable component of the phyllode protein.

Pipecolic acid degradation has been demonstrated for the first time in a biological system. The breakdown pathway was studied in *Acacia* phyllodes using H^3-pipecolic acid. Substances tentatively identified as Δ'-piperidine-2-carboxylic acid and ϵ-amino-α-hydroxycaproic acid were amongst the early degradation products. Ultimately, lysine and α-aminoadipic acid became labelled. In contrast to the experiments with C^{14}-lysine, organic acids and sugars did not become radioactive. The explanation of this finding is probably to be found in the ease with which H^3 atoms present in certain chemical groupings may undergo exchange with normal hydrogen atoms of water molecules.

The biosynthesis of 4-hydroxypipecolic acid probably involves a direct hydroxylation of the parent imino-acid.

INTRODUCTION

PIPECOLIC ACID is distributed fairly widely throughout the plant kingdom (Morrison, 1953; Grobbelaar, Zacharius, and Steward, 1954), and this imino-acid sometimes forms a major component of the soluble-nitrogen fraction of plant tissues. The formation of pipecolic acid from lysine has been demonstrated in the rat (Rothstein and Miller, 1954), in *Neurospora* mutants (Schweet, Holden and Lowy, 1954), and in beans, *Phaseolus vulgaris* (Grobbelaar and Steward, 1953; Lowy, 1953). ϵ-Amino-α-ketocaproic acid and Δ'-piperidine-2-carboxylic acid, which is formed by spontaneous cyclization of the keto-acid, are intermediates in the conversions catalysed by extracts of rat organs (Meister, 1954; Meister, Radhakrishnan, and Buckley, 1957) and *Neurospora* (Schweet, Holden, and Lowy, 1954). Δ'-Piperidine-2-carboxylic

acid is also reduced to pipecolic acid by extracts of *Pisum sativum* and *Phaseolus radiatus* in the presence of reduced diphosphopyridine nucleotide (Meister, Radhakrishnan, and Buckley, 1957). From the studies of Mann and Smithies (1955) on the amine oxidase of peas it appears that an alternative pathway for the conversion of lysine into pipecolic acid, involving the loss of the ε-amino group, may be operative in higher plants. α-Aminoadipic-δ-semialdehyde and Δ′-piperidine-6-carboxylic acid would then act as intermediates. The reverse conversion of pipecolic acid into lysine or into the intermediates could not be demonstrated with these systems. When using ε-C^{14}-lysine, the formation of labelled α-aminoadipic acid has been observed in guinea-pig liver homogenates (Borsook, Deasy, Haagen-Smit, Keighley, and Lowy, 1948), in intact rats (Rothstein and Miller, 1954), and in beans (Grobbelaar and Steward, 1953; Steward, Zacharius, and Pollard, 1955). Lysine was not converted into α-aminoadipic acid in *Neurospora* (Schweet, Holden, and Lowy, 1954). However, the reverse conversion probably occurs because growth experiments indicate that α-aminoadipic acid can satisfy the lysine requirement of certain lysineless *Neurospora* mutants (Mitchell and Houlahan, 1948). α-Aminoadipic-δ-semialdehyde has been suggested as an intermediate in the latter reaction (Schweet, Holden, and Lowy, 1954).

Rothstein and Miller (1954) have suggested that pipecolic acid is an intermediate in the formation of α-aminoadipic acid from lysine. However, no information is available to indicate whether pipecolic acid may be further degraded and so this suggestion still awaits direct experimental confirmation. One object of the present investigation was to test the biological stability of pipecolic acid and for this purpose phyllodes of *Acacia homalophylla* were used as the experimental plant material. This plant contains appreciable amounts of 4-hydroxypipecolic acid in addition to pipecolic acid, and so is like other *Acacia* species (Virtanen and Kari, 1955). The phyllodes therefore provided a tissue in which a further possible aspect of lysine and pipecolic acid metabolism could be studied. C^{14}-lysine and C^{14}- and H^3-pipecolic acids were supplied to the phyllodes and the distribution of radioactivity in other compounds was examined after various intervals of time. The results provided the first direct proof that pipecolic acid can be degraded by plants and gave some indication of the steps involved in this degradation. In addition, tentative conclusions concerning the mode of biogenesis of 5-hydroxypipecolic acid can be made.

MATERIALS AND METHODS

Isotopically labelled compounds. C^{14}-lysine, C^{14}-pipecolic acid and H^3-pipecolic acid were supplied to phyllodes in these experiments.

(i) L-C^{14}-lysine (generally labelled, specific activity 10·3 mc./mM.) was obtained from the Radiochemical Centre, Amersham.

(ii) L-C^{14}-pipecolic acid was synthesized biologically. C^{14}-lysine was supplied to *Acacia* phyllodes; 3 hours later the phyllodes were killed and the L-C^{14}-pipecolic acid formed was extracted and isolated by the methods of

paper chromatography. An outline of the procedure used is given below. Reference to the Results section shows that the C^{14}-activity appearing in pipecolic acid was at a maximum 3 hours after the initial supply of C^{14}-lysine. All the carbon atoms of the L-C^{14}-pipecolic acid isolated would be expected to be labelled equally; the specific activity was approximately 0·8 mc./mM.

(iii) H^3-pipecolic acid was prepared by tritiation of pyridine-2-carboxylic acid in the presence of Adam's platinum oxide catalyst. The apparatus used was similar to that described by Crawhall and Smyth (1958). The reduction of 11 mg. of pyridine-2-carboxylic acid, dissolved in 5 ml. dioxan, was allowed to proceed at 40° for 24 hours at low pressure (approximately 0·1 atmos. H_2 containing 94·1 per cent. H^3, total volume at N.T.P., 0·43 ml., activity 1 c.). The reduction was then completed at atmospheric pressure after introduction of ordinary hydrogen gas. Radioautographs indicated that pipecolic acid was the sole H^3-labelled product. The labile H^3 atoms attached to the nitrogen atom and the carboxyl group of pipecolic acid were replaced by hydrogen atoms by warming an aqueous solution of the labelled product for several hours. The specific activity of the pipecolic acid finally obtained was 310 mc./mM.

Plant materials. Preliminary experiments used species of two genera, *Acacia* and *Albizzia*, known to contain 4-hydroxypipecolic acid (Virtanen and Kari, 1955). C^{14}-lysine was supplied to excised leaves of *Albizzia warneka* and excised phyllodes of *Acacia homalophylla*. The pathway of lysine conversion was similar for each tissue, but breakdown was more rapid in the latter and therefore phyllodes were used in all further experiments. The phyllodes were usually immature, fresh weight approximately 0·25 g., and were taken from near a stem tip. The plants were supplied by the University of London Botanical Supply Unit, Egham.

Supply of labelled compounds. An attempt was made to supply the labelled substances to phyllode disks by vacuum infiltration, but insufficient of the solutions of radioactive acids entered the disks owing to the waxy character of their surfaces. The alternative procedure of feeding through the basal end of excised phyllodes was adopted, good lighting and ventilation being provided to increase the rates of transpiration and assimilation. Fifty microlitres of a solution of the radioactive substrate was normally supplied to each phyllode, and uptake was complete in about 2 hours. This volume of solution contained approximately 40 μg. C^{14}-lysine, 2·5 μc., 100 μg. C^{14}-pipecolic acid, 0·62 μc., and 200 μg. H^3-pipecolic acid, 500 μc. About 2 hours was required for the complete absorption of the radioactive solutions. The phyllodes were then kept at 25° under humid conditions and subjected to continuous light of 900 ft. candles. After various intervals of time, representative samples were killed by dropping into boiling 75 per cent. (v/v) ethanol.

Extraction and assay of labelled substances of phyllodes. The procedure used for the extraction of phyllodes was that described earlier for other green tissues (Fowden and Webb, 1958; Fowden and Bryant, 1959). Treatment on cation-exchange resin columns was omitted so that the distribution of

C^{14}- or H^3-label in all soluble substances, and not just in the amino-acids, could be observed. Two-dimensional paper chromatograms were prepared from portions of each concentrated extract, representing 0·1 g. fresh weight of phyllode tissue. Radioautographs of chromatograms containing C^{14}-labelled materials were obtained on Kodirex X-ray film (Fowden and Webb, 1958). When necessary, amino-acids, organic acids, and sugars were located on the chromatograms after radioautography by successive application of ninhydrin, bromophenol blue (Buch, Montgomery, and Porter, 1952), and benzidine-acetic acid (Horrocks, 1949) spraying agents. The methods used for the assay of total radioactivity in each C^{14}-labelled compound and the quantitative estimation of individual free amino-acids in the extracts followed those described in the earlier papers.

A large two-dimensional chromatogram was used to separate the biologically synthesized L-C^{14}-pipecolic acid from other radioactive substances present in the phyllode tissue (about 0·5 g.). The imino-acid was eluted from the appropriate area of filter-paper by distilled water. After evaporation, the radioactivity and pipecolic-acid content of portions of the concentrate were determined, from which the specific activity of the preparation was calculated.

Radioautographs were prepared from chromatograms containing the H^3-labelled substances by a scintillation procedure based on that of Wilson (1958). The chromatograms were placed in contact with Ilford fast blue sensitive, type XK plates and both were immersed under a 0·5 per cent. (w/v) solution of *p*-terphenyl in redistilled toluene contained in a shallow dish fitted with a ground-glass plate. The dish was then placed in a light-tight box and exposure of the plates was allowed to proceed for an appropriate period of time, usually about 6 weeks in the present experiments. The plates were rinsed in toluene and allowed to dry before development. The quality of the radioautographs was good and the spots had clear boundaries; however, background fogging was slightly more noticeable than on normal C^{14} radioautographs. The amounts of H^3 radioactivity in each soluble compound were determined using a gas flow (methane) proportional counter in a manner analogous to that used in C^{14} counting. Self-absorption by the samples war not encountered because the large amounts of H^3 activity used initially allowed samples of negligible weight (about 1 μg.) to be counted.

RESULTS

Feeding experiments with C^{14}*-lysine*

The distribution of the C^{14} atoms between various components of the aqueous-ethanol-soluble fraction of the phyllodes is illustrated by the data in Tables I and II. The incorporation of C^{14} into aqueous-ethanol-insoluble compounds was not normally studied, but the incorporation of activity into the protein fraction of the phyllodes 24 hours after the supply of C^{14}-lysine was investigated (Table III).

The total C^{14}-activity appearing in the compounds of the soluble fraction reached a maximum value 3 hours after the commencement of C^{14}-lysine

TABLE I

The distribution of radioactivity in the compounds of the 75 per cent. (v/v) ethanol-soluble fraction of Acacia *phyllodes supplied with* C^{14}*-lysine (approx. 10⁶ c.p.m./0·1 g. fresh weight plant tissue)*

(The activity present in each compound is expressed as a percentage of the total activity in the soluble fraction)

	Hours after supplying C^{14}-lysine to the phyllodes						
	1·5	3	6	12	24	48	120
Total activity in soluble compounds (c.p.m. x 10^{-3}/0·1 g. F.W. tissue)	362	664	400	266	202	204	182
Lysine	60·2	21·1	14·7	12·8	16·0	5·3	0·8
Pipecolic acid	24·5	42·2	47·9	37·7	41·5	39·9	38·2
4-Hydroxypipecolic acid	0·7	0·7	0·9	1·1	2·1	2·4	2·7
α-Aminoadipic acid	3·0	3·5	2·1	1·9	1·2	0·8	—
Glutamic acid	2·0	13·4	9·6	8·4	6·8	2·6	0·5
Proline	2·0	3·8	4·2	4·6	4·4	3·2	4·6
Glutamine	0·4	2·6	1·8	3·3	5·1	2·3	0·8
Aspartic acid	—	0·7	0·2	0·3	0·4	—	—
Sucrose	—	1·7	4·5	9·1	10·6	24·7	26·3
Glucose	—	—	—	—	—	—	4·4
Fructose	—	—	—	—	—	—	5·4
Malic acid	—	2·0	1·7	5·6	6·4	14·0	13·2
Citric acid	0·8	0·6	0·6	0·7	0·4	0·6	1·3
Unknown *B**	2·9	4·4	8·0	9·0	1·8	—	—
Unknown *C**	—	1·8	1·4	3·9	1·7	2·7	—
Unknown *D**	3·5	1·6	2·3	1·7	1·5	1·5	1·9

*On two-dimensional chromatograms (phenol-NH_3/butanol-acetic acid-water), unknown *B* moves to a position close to that of leucine, unknown *C* is near valine, and unknown *D* is near asparagine.

feeding. The activity in the soluble compounds then represented 56 per cent. of that supplied initially as C^{14}-lysine. The values obtained at 1·5 hours are not strictly comparable with those at later times because absorption of the radioactive substrates by the phyllodes normally required about 2 hours. A progressive decrease in the activity present in the soluble compounds occurred as the metabolic period was increased beyond 3 hours. At 24 hours, the total activity determined in soluble compounds and in protein represented only 31 per cent. of that initially supplied; about 48 per cent. of the C^{14}-activity had been liberated as respiratory carbon dioxide at this time. The production of $C^{14}O_2$ is indicative that the carbon skeleton of lysine was metabolized extensively, and this conclusion is confirmed by reference to Table I, which shows that C^{14}-activity quickly entered a wide variety of compounds including amino-acids, organic acids, sugars and other unidentified substances.

The outstanding feature of lysine metabolism in the phyllodes is the degree to which it is converted to pipecolic acid. This imino-acid rapidly acquired more C^{14}-activity than that remaining in lysine, and after only 3 hours it contained 42 per cent. of the total radioactivity in soluble compounds (or

TABLE II

The specific activities of certain free amino-acids present in Acacia *phyllodes after supplying* C^{14}*-lysine of specific activity* 8×10^5 *c.p.m./μg.* α-NH_2-N

(*Data are expressed as c.p.m./μg.* α-NH_2-N *present in each amino-acid*)

	Hours after supplying C^{14}-lysine to the phyllodes						
	1·5	3	6	12	24	48	120
Lysine	108,000	70,100	58,800	42,500	32,300	10,800	3,620
Pipecolic acid	8,850	28,000	19,200	10,000	10,400	8,120	8,710
4-Hydroxypipecolic acid	310	452	543	494	708	824	618
α-Aminoadipic acid	26,800	57,700	21,300	12,600	12,300	4,350	—
Glutamic acid	1,260	14,800	8,600	4,460	2,300	1,300	210
Proline	785	6,350	5,630	4,130	2,200	2,180	2,070
Glutamine	492	3,500	2,430	2,180	2,060	1,560	473
Aspartic acid	—	4,450	761	389	260	—	—

TABLE III

The distribution of radioactivity between certain amino-acids present in the protein of Acacia *phyllodes 24 hours after supplying* C^{14}*-lysine of specific activity* 8×10^5 *c.p.m./μg.* α-NH_2-N

	Activity in amino-acids present in 1 mg. protein† (c.p.m.)	Activity in amino-acids as % of total activity in protein	g. α-NH_2-N in amino-acid/100 g. protein-N	Specific activity of amino-acids (c.p.m./μg. α-NH_2-N)
Lysine	120,800	53·7	4·5	16,800
Glutamic acid	58,700	26·1	8·5	4,330
Proline	19,600	8·7	5·2	2,360
Aspartic acid	6,520	2·9	6·9	593
Hydroxyproline	5,400	2·4	2·9	1,080
Alanine	4,280	1·9	7·1	375
α-Aminoadipic acid	2,930	1·3	0·5	3,660
Phenylalanine	2,700	1·2	2·5	675
Unknown*	4,050	1·8	—	—

* The unknown amino-acid has the following Rf values: Phenol-NH_3, 0·51; *n*-butanol-acetic acid-water (4:1:5), 0·01.

† Calculated assuming the protein contains 16% nitrogen.

28 per cent. of the C^{14}-activity initially supplied as lysine). Although pipecolic acid continued to represent a major proportion of the total radioactivity of the soluble compounds, the activity present in the imino-acid slowly decreased at subsequent times of analysis. The specific activity of the pipecolic acid was also highest at 3 hours. Glutamic and aspartic acids, proline and α-aminoadipic acid also showed their maximum specific activities 3 hours after supply of C^{14}-lysine. Although the total amount of C^{14}-activity in α-aminoadipic acid was quite small, the specific activity of this amino-acid exceeded that of all others, including pipecolic acid.

C^{14}-activity entered into 4-hydroxypipecolic acid more slowly than into the other amino-acids investigated. The hydroxy compound only gained its

maximum specific activity 48 hours after the supply of C^{14}-lysine was commenced.

Other aqueous-ethanol-soluble compounds into which radioactivity was introduced quickly included malic and citric acids, sucrose, and the unidentified compounds *B*, *C*, and *D*. The total C^{14}-activity present in sucrose and malic acid increased continuously with time until, at 120 hours, they represented important percentages of the total radioactivity present in soluble compounds.

The distribution of radioactivity between the amino-acids present in the protein isolated from phyllodes 24 hours after the supply of C^{14}-lysine is shown in Table III. Lysine was labelled most heavily; glutamate and proline were also strongly marked. Several other amino-acids, including α-aminoadipic acid, contained small amounts of C^{14}. The specific activities of the different acids followed much the same pattern; however, that of α-aminoadipic acid was relatively high, owing to the small quantity of the acid present in the protein. In certain cases, for example glutamate, aspartate, and proline, the specific activity of the protein-bound amino-acid was higher than that determined at 24 hours for the corresponding free amino-acid.

Feeding experiments with C^{14}*-pipecolic acid*

The distribution of radioactivity between the aqueous-ethanol-soluble compounds of phyllodes was examined 5 and 24 hours after supplying C^{14}-pipecolic acid.

The radioautograph prepared from the extract of phyllodes killed after 5 hours showed only two faint spots together with an intense spot corresponding to unchanged pipecolic acid. One of the weakly radioactive spots occupied the same position on the two-dimensional chromatogram as ε-amino-α-hydroxycaproic acid. The identity of the other faint spot remains unknown. The amounts of C^{14}-activity in these two degradation products was too small for accurate assay.

Twenty-four hours after supplying C^{14}-pipecolic acid to the phyllodes, the C^{14}-label was more widely distributed and was found in various amino-acids, organic acids and sucrose (Table IV). About 33 per cent. of the total activity supplied as C^{14}-pipecolic acid was accounted for by these components of the aqueous-ethanol-soluble fraction; respiratory carbon dioxide accounted for a further 39 per cent. of the original activity. C^{14}-activity in pipecolic acid still represented over half the total activity of the soluble compounds. Lysine appeared amongst the labelled products of metabolism and its specific activity was higher than those of α-aminoadipic acid or glutamic acid. After 24 hours the substance tentatively identified as ε-amino-α-hydroxycaproic acid contained only a very small fraction of the total radioactivity. It was impossible to estimate its specific activity because the quantities present in the phyllodes were too small to assay by the ninhydrin technique. At this time 4-hydroxypipecolic acid was also labelled, its specific activity being approximately

one-tenth of that of pipecolic acid. No radioactive areas were visible on the radioautograph in positions associated with hydroxylysines.

TABLE IV

The distribution of radioactivity in the compounds of the 75 per cent. (v/v) ethanol-soluble fraction of Acacia *phyllodes 24 hours after supplying* C^{14}*-pipecolic acid*[•] *(approx.* $2{\cdot}5 \times 10^5$ *c.p.m./0·1 g. fresh-weight plant tissue)*

	Total activity (c.p.m./0·1 g. F.Wt. material)	Activity as % of total soluble activity	Specific activity of amino-acids (c.p.m./μg. α-NH_2-N)
Pipecolic acid . . .	45,500	55·0	11,400
Lysine	2,625	3·2	4,370
α-Aminoadipic acid . .	2,220	2·7	3,700
Glutamic acid . . .	7,540	9·1	2,510
Glutamine . . .	3,850	4·7	1,540
Proline	2,410	2·9	1,205
4-Hydroxypipecolic acid .	3,370	4·1	1,120
ε-Amino-α-ketocaproic acid .	985	1·2	—
Sucrose	5,260	6·4	—
Citric acid . . .	6,750	8·2	—
Malic acid . . .	2,120	2·5	—

[•] The specific activity of the pipecolic acid was approx. $5{\cdot}8 \times 10^4$ c.p.m./μg. imino-N.

Feeding experiments with H^3*-pipecolic acid*

The progressive degradation with time of H^3-pipecolic acid supplied to the phyllodes is illustrated by the reproductions of the radioautographs shown in Fig. 1, and by the data in Table V. The assimilation of the H^3-pipecolic acid was not quite complete at 2 hours and so the total activity present in the soluble compounds at this time was less than that determined after 4 hours. Although the soluble compounds attain their maximum degree of labelling at 4 hours, their total activity only represented approximately half of the activity supplied as pipecolic acid. After 96 hours, about 10 per cent. of the H^3 supplied was still present in the soluble fraction. The degree to which H^3 was lost from the soluble fraction during a 24-hour metabolic period (presumably it would be predominantly incorporated into water molecules) was greater than the observed rate of disappearance of C^{14} from this fraction in the C^{14}-pipecolic acid experiments (Table IV).

Tritium rapidly accumulated in a compound *E* that was not completely separated from pipecolic acid on the chromatograms (Fig. 1). *E* did not give a ninhydrin colour. Its chromatographic position is very similar to that occupied Δ′-piperidine-2-carboxylic acid (prepared from ε-carbobenzoxy-α-ketocaproic acid, generously supplied by Dr. A. Meister (see Meister, 1954)). Other early degradation products included 4-hydroxypipecolic acid and ε-amino-α-hydroxycaproic acid (spots *F* and *G* respectively on Fig. 1, (*a*) and (*b*)). Lysine, and α-aminoadipic and glutamic acids subsequently became

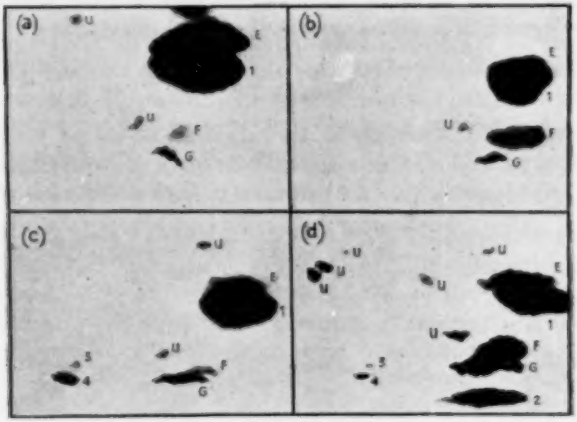


FIG. 1. Reproductions of radioautographs prepared from extracts of *Acacia* phyllodes supplied with H^3-pipecolic acid. The phyllodes were extracted (*a*) 4 hours, (*b*) 8 hours, (*c*) 24 hours, and (*d*) 48 hours after commencing pipecolic acid feeding. The extracts were applied at the bottom left-hand corner of the chromatograms, which were developed in phenol-NH_3 (flow along horizontal direction) followed by *n*-butanol-acetic acid-water mixture (vertical). The radioactive spots were identified as: 1, pipecolic acid; 2, lysine; 3, α-aminoadipic acid; 4, glutamic acid; *E*, Δ′-piperidine-2-carboxylic acid; *F*, 4-hydroxypipecolic acid; *G*, ε-amino-α-hydroxycaproic acid; *U*, unknown compounds.

TABLE V

The distribution of radioactivity amongst the compounds of the 75 per cent.* (v/v) *ethanol-soluble fraction of* Acacia *phyllodes after supplying* H^3*-pipecolic acid (approx.* 2×10^8 *c.p.m./0·1 g. fresh weight plant tissue)

(*The activity present in each compound is expressed as counts per min.* $\times 10^{-4}$*/0·1 g. fresh weight plant material*)

	Hours after supplying H^3-pipecolic acid to the phyllodes					
	2	4	8	24	48	96
Total activity in soluble compounds	71·2	93·8	35·6	40·7	29·9	23·6
Pipecolic acid	650·0	810·0	327·0	360·0	237·0	191·0
Δ′-piperidine-2-carboxylic acid (*E*)	51·4	112·0	13·0	19·1	29·5	23·1
4-Hydroxypipecolic acid	3·4	5·7	10·2	14·1	8·9	6·2
ε-Amino-α-hydroxycaproic acid (*G*)	5·0	7·0	4·4	5·7	10·2	5·2
Glutamic acid	—	—	—	3·8	4·3	2·1
α-Aminoadipic acid	—	—	—	1·0	0·6	—
Lysine	—	—	—	1·0	5·8	6·0

radioactive. Specific activities of the amino-acids were not determined in this experiment because the acids that became rapidly labelled were present in amounts too low to assay. While a number of labelled products remained unidentified (spots designated *U* in Fig. 1), it is noteworthy that no activity

was detected in sucrose, glucose, or fructose, or in citric or malic acids in any of the samples analysed.

DISCUSSION

Previous studies of the metabolism of C^{14}-lysine by intact plant organs appear to be confined to the single species, *Phaseolus vulgaris* (Grobbelaar and Steward, 1953; Lowy, 1953). Although the metabolism of the lysine supplied was allowed to proceed for one or more days, the C^{14}-label was incorporated into only a few aqueous-ethanol-soluble compounds. Pipecolic acid was the predominant radioactive product, but α-aminoadipic acid and one other unidentified compound also became labelled in the experiments of Steward (Steward, Zacharius, and Pollard, 1955). A similar restricted metabolism of C^{14}-lysine has been reported by Schweet, Holden, and Lowy (1954) during the growth of *Neurospora* for a period of 5 days. Pipecolic acid, together with a derivative of ε-amino-α-hydroxycaproic acid, were the only soluble compounds to become radioactive. The present findings using *Acacia* phyllodes are in sharp contrast to these earlier experiments because C^{14} atoms supplied initially as lysine were distributed widely, not only in amino-acids, but also in various organic acids and sugars. The extensive degradation of lysine by *Acacia* phyllodes is reminiscent of the fate of the labelled amino-acid after supplying to intact rats (Rothstein and Miller, 1954).

Biodegradation of pipecolic acid has also been demonstrated for the first time. Although C^{14} atoms supplied as pipecolic acid are incorporated into other soluble compounds more slowly than similar atoms supplied as lysine, ultimately a number of compounds became radioactive. As mentioned earlier, the range of compounds becoming labelled in the H^3-pipecolic acid experiments was more restricted than that observed after supplying C^{14}-pipecolic acid. An explanation of this observation is to be found in the known chemical lability of H^3 atoms attached to certain chemical groupings. Tritium atoms present in amino, carboxyl, and hydroxyl groups undergo a rapid exchange reaction with normal hydrogen atoms of water, whilst those attached to carbon atoms immediately adjacent to carbonyl or carboxyl groups take part in similar, but slower, exchanges under certain conditions. As the degradation of H^3-pipecolic acid becomes more extensive, the intermediate breakdown products contain more and more of the H^3 atoms in such labile positions, so that a state is finally reached in which intermediates, only distantly related to pipecolic acid, are no longer radioactive. The experimental results indicate that this loss of H^3 atoms may become essentially complete during the metabolism of the tricarboxylic acid cycle intermediates. From the experimental results, which indicate that glutamate present in the phyllodes was labelled, whereas no activity was found in aspartate or alanine, the loss of H^3 atoms apparently became virtually complete between the stages of α-ketoglutarate and oxaloacetate or pyruvate.

Some tentative conclusions concerning the metabolic pathways involved in C^{14}-lysine degradation can be formulated from the specific activity data

maximum specific activity 48 hours after the supply of C^{14}-lysine was commenced.

Other aqueous-ethanol-soluble compounds into which radioactivity was introduced quickly included malic and citric acids, sucrose, and the unidentified compounds *B*, *C*, and *D*. The total C^{14}-activity present in sucrose and malic acid increased continuously with time until, at 120 hours, they represented important percentages of the total radioactivity present in soluble compounds.

The distribution of radioactivity between the amino-acids present in the protein isolated from phyllodes 24 hours after the supply of C^{14}-lysine is shown in Table III. Lysine was labelled most heavily; glutamate and proline were also strongly marked. Several other amino-acids, including α-aminoadipic acid, contained small amounts of C^{14}. The specific activities of the different acids followed much the same pattern; however, that of α-aminoadipic acid was relatively high, owing to the small quantity of the acid present in the protein. In certain cases, for example glutamate, aspartate, and proline, the specific activity of the protein-bound amino-acid was higher than that determined at 24 hours for the corresponding free amino-acid.

Feeding experiments with C^{14}-pipecolic acid

The distribution of radioactivity between the aqueous-ethanol-soluble compounds of phyllodes was examined 5 and 24 hours after supplying C^{14}-pipecolic acid.

The radioautograph prepared from the extract of phyllodes killed after 5 hours showed only two faint spots together with an intense spot corresponding to unchanged pipecolic acid. One of the weakly radioactive spots occupied the same position on the two-dimensional chromatogram as ε-amino-α-hydroxycaproic acid. The identity of the other faint spot remains unknown. The amounts of C^{14}-activity in these two degradation products was too small for accurate assay.

Twenty-four hours after supplying C^{14}-pipecolic acid to the phyllodes, the C^{14}-label was more widely distributed and was found in various amino-acids, organic acids and sucrose (Table IV). About 33 per cent. of the total activity supplied as C^{14}-pipecolic acid was accounted for by these components of the aqueous-ethanol-soluble fraction; respiratory carbon dioxide accounted for a further 39 per cent. of the original activity. C^{14}-activity in pipecolic acid still represented over half the total activity of the soluble compounds. Lysine appeared amongst the labelled products of metabolism and its specific activity was higher than those of α-aminoadipic acid or glutamic acid. After 24 hours the substance tentatively identified as ε-amino-α-hydroxycaproic acid contained only a very small fraction of the total radioactivity. It was impossible to estimate its specific activity because the quantities present in the phyllodes were too small to assay by the ninhydrin technique. At this time 4-hydroxypipecolic acid was also labelled, its specific activity being approximately

one-tenth of that of pipecolic acid. No radioactive areas were visible on the radioautograph in positions associated with hydroxylysines.

TABLE IV

The distribution of radioactivity in the compounds of the 75 per cent. (v/v) ethanol-soluble fraction of Acacia *phyllodes 24 hours after supplying* C^{14}*-pipecolic acid* (approx.* $2{\cdot}5 \times 10^5$ *c.p.m./0·1 g. fresh-weight plant tissue)*

	Total activity (c.p.m./0·1 g. F.Wt. material)	Activity as % of total-soluble activity	Specific activity of amino-acids (c.p.m./μg. α-NH_2-N)
Pipecolic acid	43,500	55·0	11,400
Lysine	2,625	3·2	4,370
α-Aminoadipic acid	2,220	2·7	3,700
Glutamic acid	7,540	9·1	2,510
Glutamine	3,850	4·7	1,540
Proline	2,410	2·9	1,205
4-Hydroxypipecolic acid	3,370	4·1	1,120
ε-Amino-α-ketocaproic acid	985	1·2	—
Sucrose	5,260	6·4	—
Citric acid	6,750	8·2	—
Malic acid	2,120	2·5	—

* The specific activity of the pipecolic acid was approx. $5{\cdot}8 \times 10^4$ c.p.m./μg. imino-N.

Feeding experiments with H^3*-pipecolic acid*

The progressive degradation with time of H^3-pipecolic acid supplied to the phyllodes is illustrated by the reproductions of the radioautographs shown in Fig. 1, and by the data in Table V. The assimilation of the H^3-pipecolic acid was not quite complete at 2 hours and so the total activity present in the soluble compounds at this time was less than that determined after 4 hours. Although the soluble compounds attain their maximum degree of labelling at 4 hours, their total activity only represented approximately half of the activity supplied as pipecolic acid. After 96 hours, about 10 per cent. of the H^3 supplied was still present in the soluble fraction. The degree to which H^3 was lost from the soluble fraction during a 24-hour metabolic period (presumably it would be predominantly incorporated into water molecules) was greater than the observed rate of disappearance of C^{14} from this fraction in the C^{14}-pipecolic acid experiments (Table IV).

Tritium rapidly accumulated in a compound *E* that was not completely separated from pipecolic acid on the chromatograms (Fig. 1). *E* did not give a ninhydrin colour. Its chromatographic position is very similar to that occupied Δ′-piperidine-2-carboxylic acid (prepared from ε-carbobenzoxy-α-ketocaproic acid, generously supplied by Dr. A. Meister (see Meister, 1954)). Other early degradation products included 4-hydroxypipecolic acid and ε-amino-α-hydroxycaproic acid (spots *F* and *G* respectively on Fig. 1, (*a*) and (*b*)). Lysine, and α-aminoadipic and glutamic acids subsequently became

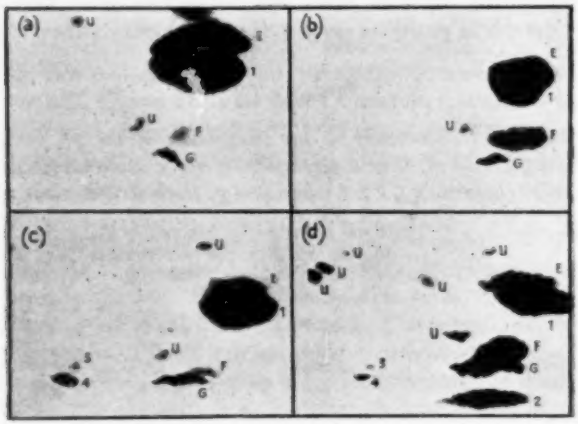


FIG. 1. Reproductions of radioautographs prepared from extracts of *Acacia* phyllodes supplied with H^3-pipecolic acid. The phyllodes were extracted (*a*) 4 hours, (*b*) 8 hours, (*c*) 24 hours, and (*d*) 48 hours after commencing pipecolic acid feeding. The extracts were applied at the bottom left-hand corner of the chromatograms, which were developed in phenol-NH_3 (flow along horizontal direction) followed by *n*-butanol-acetic acid-water mixture (vertical). The radioactive spots were identified as: 1, pipecolic acid; 2, lysine; 3, α-aminoadipic acid; 4, glutamic acid; *E*, Δ′-piperidine-2-carboxylic acid; *F*, 4-hydroxypipecolic acid; *G*, ε-amino-α-hydroxycaproic acid; *U*, unknown compounds.

TABLE V

The distribution of radioactivity amongst the compounds of the 75 per cent. (*v*/*v*) *ethanol-soluble fraction of* Acacia *phyllodes after supplying* H^3*-pipecolic acid* (*approx.* 2×10^8 *c.p.m./0·1 g. fresh weight plant tissue*)

(*The activity present in each compound is expressed as counts per min.* $\times 10^{-3}$*/0·1 g. fresh weight plant material*)

	Hours after supplying H^3-pipecolic acid to the phyllodes					
	2	4	8	24	48	96
Total activity in soluble compounds	71·2	93·8	35·6	40·7	29·9	23·6
Pipecolic acid	650·0	810·0	327·0	360·0	237·0	191·0
Δ′-piperidine-2-carboxylic acid (*E*)	51·4	112·0	13·0	19·1	29·5	23·1
4-Hydroxypipecolic acid	3·4	5·7	10·2	14·1	8·9	6·2
ε-Amino-α-hydroxycaproic acid (*G*)	5·0	7·0	4·4	5·7	10·2	5·2
Glutamic acid	—	—	—	3·8	4·3	2·1
α-Aminoadipic acid	—	—	—	1·0	0·6	—
Lysine	—	—	—	1·0	5·8	6·0

radioactive. Specific activities of the amino-acids were not determined in this experiment because the acids that became rapidly labelled were present in amounts too low to assay. While a number of labelled products remained unidentified (spots designated *U* in Fig. 1), it is noteworthy that no activity

was detected in sucrose, glucose, or fructose, or in citric or malic acids in any of the samples analysed.

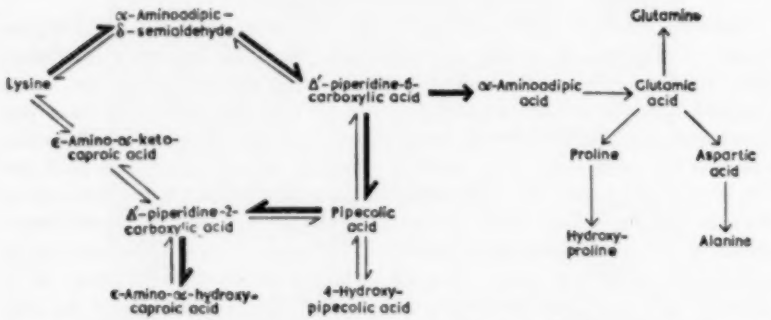
DISCUSSION

Previous studies of the metabolism of C^{14}-lysine by intact plant organs appear to be confined to the single species, *Phaseolus vulgaris* (Grobbelaar and Steward, 1953; Lowy, 1953). Although the metabolism of the lysine supplied was allowed to proceed for one or more days, the C^{14}-label was incorporated into only a few aqueous-ethanol-soluble compounds. Pipecolic acid was the predominant radioactive product, but α-aminoadipic acid and one other unidentified compound also became labelled in the experiments of Steward (Steward, Zacharius, and Pollard, 1955). A similar restricted metabolism of C^{14}-lysine has been reported by Schweet, Holden, and Lowy (1954) during the growth of *Neurospora* for a period of 5 days. Pipecolic acid, together with a derivative of ε-amino-α-hydroxycaproic acid, were the only soluble compounds to become radioactive. The present findings using *Acacia* phyllodes are in sharp contrast to these earlier experiments because C^{14} atoms supplied initially as lysine were distributed widely, not only in amino-acids, but also in various organic acids and sugars. The extensive degradation of lysine by *Acacia* phyllodes is reminiscent of the fate of the labelled amino-acid after supplying to intact rats (Rothstein and Miller, 1954).

Biodegradation of pipecolic acid has also been demonstrated for the first time. Although C^{14} atoms supplied as pipecolic acid are incorporated into other soluble compounds more slowly than similar atoms supplied as lysine, ultimately a number of compounds became radioactive. As mentioned earlier, the range of compounds becoming labelled in the H^3-pipecolic acid experiments was more restricted than that observed after supplying C^{14}-pipecolic acid. An explanation of this observation is to be found in the known chemical lability of H^3 atoms attached to certain chemical groupings. Tritium atoms present in amino, carboxyl, and hydroxyl groups undergo a rapid exchange reaction with normal hydrogen atoms of water, whilst those attached to carbon atoms immediately adjacent to carbonyl or carboxyl groups take part in similar, but slower, exchange reactions under certain conditions. As the degradation of H^3-pipecolic acid becomes more extensive, the intermediate breakdown products contain more and more of the H^3 atoms in such labile positions, so that a state is finally reached in which intermediates, only distantly related to pipecolic acid, are no longer radioactive. The experimental results indicate that this loss of H^3 atoms may become essentially complete during the metabolism of the tricarboxylic acid cycle intermediates. From the experimental results, which indicate that glutamate present in the phyllodes was labelled, whereas no activity was found in aspartate or alanine, the loss of H^3 atoms apparently became virtually complete between the stages of α-ketoglutarate and oxaloacetate or pyruvate.

Some tentative conclusions concerning the metabolic pathways involved in C^{14}-lysine degradation can be formulated from the specific activity data

presented in Table II, and the scheme below would seem to satisfy the more important experimental findings;



The experimental results suggest that the reactions indicated by heavy-type arrows proceed more rapidly than those of alternate pathways.

The scheme suggests that lysine and pipecolic acid may be interconverted by two different pathways. However, the observation that, 3 hours after supplying C^{14}-lysine to *Acacia* phyllodes, the specific activity of α-aminoadipic acid was greater than that of pipecolic acid (Table II) indicated that the acidic amino-acid was not formed primarily from the imino-acid by the pathway operative in the intact rat as suggested by Rothstein and Miller (1954), but from α-aminoadipic-δ-semialdehyde (the open chain form of Δ′-piperidine-6-carboxylic acid), formed directly from the lysine. Whilst specific activity data are notoriously difficult to interpret owing to the varying degrees of isotopic dilution produced by the existing unlabelled molecules of different metabolites, the evidence available from the *Acacia* phyllode experiments suggests that pipecolic acid may be formed primarily from lysine, via Δ′-piperidine-6-carboxylic acid and α-aminoadipic-δ-semialdehyde. The observations do not, however, discount the possibility that some pipecolic acid is formed from lysine via Δ′-piperidine-2-carboxylic acid and ε-amino-α-keto-caproic acid. However, the former set of reactions producing pipecolic acid are considered to proceed more rapidly than the latter.

The experimental observations concerning the degradation of pipecolic acid suggest that ε-amino-α-ketocaproic acid is formed more rapidly than the alternative possibility, α-aminoadipic-δ-semialdehyde. This conclusion is supported by the finding that ε-amino-α-hydroxycaproic acid became radioactive during the metabolism of H^3-pipecolic acid at a much earlier time than did α-aminoadipic acid. The fact that the unidentified radioactive compound *E* (Fig. 1) moved to a position identical with that of Δ′-piperidine-2-carboxylic acid on paper chromatograms provides further support for this mechanism of pipecolic acid degradation. However, it should be noted that no information is available concerning the chromatographic behaviour of Δ′-piperidine-

6-carboxylic acid. Although information obtained in the C^{14}-pipecolic acid degradation experiments was very limited, ϵ-amino-α-hydroxycaproic acid again appeared as one of two radioactive degradation products formed after 5 hours metabolism by the *Acacia* phyllodes. Lysine and α-aminoadipic acid first became radioactive 24 hours after supplying H^3-pipecolic acid, and therefore the amination of the ϵ-amino-α-ketocaproic acid and the oxidation of pipecolic acid via the intermediate aldehyde to α-aminoadipic acid are both viewed as relatively slow reaction processes.

The metabolism of this family of lysine derivatives as observed in *Acacia* phyllodes appears to have certain distinct differences from that observed either in the intact rat (Rothstein and Miller, 1954) or in the fungus *Neurospora* (Schweet, Holden, and Lowy, 1954). In both of the latter systems the conversion of lysine into pipecolic acid was regarded as being irreversible, but in *Acacia* phyllodes the reaction has been shown to be readily reversed in experiments using either C^{14}- of H^3-labelled pipecolic acids. This variation of metabolic pattern between different species has a precedent in other aspects of lysine metabolism. In micro-organisms, α-aminoadipic acid may be converted into lysine, as indicated by the growth-requirement experiments of Mitchell and Houlahan (1948) using *Neurospora crassa*, and by the more direct enzymic reduction of α-aminoadipic acid catalysed by an enzyme preparation from *Torulopsis utilis* (Sagisaka and Shimura, 1959), but Rothstein and Miller (1954) concluded that α-aminoadipic acid could not be converted into lysine in the rat. A further species difference can be seen in relation to pipecolic acid metabolism. Whereas the imino-acid has been shown to be converted into α-aminoadipic acid in *Acacia* phyllodes, and the same conversion is considered to occur in the rat (Rothstein and Miller, 1954), this conversion could not be demonstrated in *Neurospora* (Schweet, Holden, and Lowy, 1954).

Two general mechanisms can be postulated for the biogenesis of hydroxyimino acids. The first proposes that the heterocyclic ring is formed only after the hydroxyl group has been introduced into an appropriate open-chain amino-acid, which, in the case of an hydroxypipecolic acid, would be lysine. For the formation of 4-hydroxypipecolic acid, γ-hydroxylysine must be formed first if this mechanism is operative. However, there is as yet no report concerning the natural occurrence of this hydroxyamino acid. In the second mechanism, the hydroxyl group is introduced after the formation of the heterocyclic ring. It is this latter mechanism that is known to be responsible for the formation of hydroxyproline in animal tissues (Stetten and Schoenheimer, 1944), and in tissue cultures of carrot root (Pollard and Steward, 1959), where hydroxyproline is produced by the hydroxylation of proline only after the latter imino-acid has been incorporated into the cell protein. The experimental evidence now presented indicates that this second mechanism is probably the one operative in the production of 4-hydroxypipecolic acid by *Acacia* phyllodes. In the H^3-pipecolic acid feeding experiments, the hydroxyimino acid became radioactive far earlier than lysine, and no trace of any radioactive substance possessing chromatographic properties akin to those

that might be expected for hydroxylysines was observed. The experiments in which C^{14}-lysine was supplied to the phyllodes did not provide such useful information regarding the manner of 4-hydroxypipecolic acid biosynthesis because a far greater number of other amino-acids attained their maximum degree of labelling considerably earlier than did the hydroxyimino acid. While the total activity present in 4-hydroxypipecolic acid increased continually up to 120 hours after the initial supply of C^{14}-lysine, the compound acquired its maximum activity after 24 hours in the H^3-pipecolic acid experiments. The observation that the radioactivity associated with 4-hydroxypipecolic acid decreased after 24 hours in the latter experiments suggested that it may be degraded by the *Acacia* phyllodes.

The present experiments do not provide any precise information regarding the metabolic pathway followed by the carbon skeleton of lysine beyond the stage of α-aminoadipic acid. The specific activity data presented in Tables II and III suggest that glutamic acid is formed before a number of other amino-acids normally present in protein. Rothstein and Miller (1954*a*) obtained evidence suggesting that α-aminoadipic acid underwent oxidative deamination and subsequent decarboxylation to yield glutaric acid, which was then further metabolized via α-hydroxyglutarate to give α-ketoglutaric acid. Similar mechanisms may be operative in the *Acacia* phyllodes producing α-ketoglutarate, subsequently aminated to glutamic acid. The specific activities observed for the other protein amino-acids are in agreement with the orders suggested for their formation in the scheme above, which has gained widespread support from numerous investigations with different plant species. It is of interest to note that the protein present in the *Acacia* phyllodes contains a considerable proportion of hydroxyproline, which is a relatively rare component of plant proteins.

The specific activities of many of the amino-acids present in the proteins of the phyllodes 24 hours after the supply of C^{14}-lysine were greater than those of the corresponding free amino-acids. This observation may be explained by assuming that, once an amino-acid molecule has been incorporated into protein, the possibility that it will undergo further degradative metabolism is considerably reduced. Therefore it is not surprising to find that this difference is most marked for a substance like glutamic acid, which is known to be particularly active in intermediary metabolic systems.

The H^3-label has been found particularly useful in the present studies. Synthesis of H^3-pipecolic acid by tritiation of pyridine-2-carboxylic acid provided a cheap product of high specific activity. Since lysine and pipecolic acid may be precursors of certain alkaloids such as anabasine and coniine (see review, Mothes, 1959), H^3-labelled compounds may prove equally useful in studies involving piperidine-type alkaloids.

ACKNOWLEDGEMENTS

The author is grateful to Miss J. Chessun for expert technical assistance during the course of this investigation, and to the Rockefeller Foundation and

the Central Research Funds Committee of the University of London for grants in support of the work.

LITERATURE CITED

BORSOOK, H., DEASY, C. L., HAAGEN-SMIT, A. J., KEIGHLEY, G., and LOWY, P. H. (1948). *J. Biol. Chem.* **171**, 255.

BUCH, M. L., MONTGOMERY, R., and PORTER, W. L. (1952). *Anal. Chem.* **24**, 489.

CRAWHALL, J. C., and SMYTH, D. G. (1958). *Biochem. J.* **69**, 280.

FOWDEN, L., and BRYANT, M. (1959). Ibid. **71**, 210.

—— and WEBB, J. A. (1958). *Ann. Bot.*, N.S. **22**, 73.

GROBBELAAR, N., and STEWARD, F. C. (1953). *J. Amer. Chem. Soc.* **75**, 4341.

—— ZACHARIUS, R. M., and STEWARD, F. C. (1954). Ibid. **76**, 2912.

HORROCKS, R. H. (1949). *Nature*, Lond. **164**, 444.

LOWY, P. H. (1953). *Arch. Biochem. Biophys.* **47**, 228.

MANN, P. J. G., and SMITHIES, W. R. (1955). *Biochem. J.* **61**, 89.

MEISTER, A. (1954). *J. Biol. Chem.* **206**, 577.

—— RADHAKRISHNAN, A. N., and BUCKLEY, S. D. (1957). Ibid. **229**, 789.

MITCHELL, H. K., and HOULAHAN, M. B. (1948). Ibid. **174**, 883.

MORRISON, R. I. (1953). *Biochem. J.* **53**, 474.

MOTHES, K. (1959). *Soc. Exp. Biol. Symposia*, **13**, 258.

POLLARD, J. K., and STEWARD, F. C. (1959). *J. Exp. Bot.* **10**, 17.

ROTHSTEIN, M., and MILLER, L. L. (1954). *J. Biol. Chem.* **211**, 851.

—— —— (1954 *a*). Ibid. **211**, 859.

SAGISAKA, S., and SHIMURA, K. (1959). *Nature*, Lond. **184**, 1709.

SCHWEET, R. S., HOLDEN, J. T., and LOWY, P. H. (1954). *J. Biol. Chem.* **211**, 517.

STETTEN, M. R., and SCHOENHEIMER, R. (1944). Ibid. **153**, 113.

STEWARD, F. C., ZACHARIUS, R. M., and POLLARD, J. K. (1955). *Ann. Acad. Sci. Fenn.* (*Ser. A II, Chem.*), **60**, 321.

VIRTANEN, A. I., and KARI, S. (1955). *Acta Chem. Scand.* **9**, 170.

WILSON, A. T. (1958). *Nature*, Lond. **182**, 524.

The Organic Acid Metabolism of Cox's Orange Pippin Apples

I. SOME EFFECTS OF THE ADDITION OF ORGANIC ACIDS TO THE PEEL OF THE FRUIT

A. E. FLOOD

East Malling Research Station, Maidstone, Kent

A. C. HULME AND L. S. C. WOOLTORTON

Ditton Laboratory, Larkfield, Maidstone, Kent

Received 29 February 1960

SUMMARY

1. The basic respirations (CO_2-output and O_2-uptake) of Cox's Orange Pippin apples and of the peel tissue prepared from them were compared in fruit in various stages of development, both initially and after storage at 12° C. Both show the same general trend, although as the apples become mature the peak value of the respiration climacteric tends to rise in the whole fruit and fall in the peel.

2. The effect of adding malate or citrate on the respiration of the same samples of peel was studied.

3. Three broad stages of development were observed. During the first stage (petal fall to 60 days after) the metabolic pattern appears to be different from the two later stages. Here O_2-uptake as well as CO_2-output are influenced by the addition of both malate and, to a considerably less extent, citrate. In stage 2 (60–125 days from petal fall), the malate effect (CO_2-output) is small until after detachment from the tree, when it rises sharply. In stage 3 (125 days to full maturity) the malate effect follows the course expected for earlier work, namely, it develops at the same time as the climacteric rise in respiration. The possible reasons for the different behaviour of the peel at the three stages is discussed.

4. Results were similar in general trend for Cox's Orange Pippin apples grown on different rootstocks and under different cultural conditions.

5. It is suggested that the malate effect is most active in the epidermal and hypodermal tissues of the fruit.

INTRODUCTION

It has already been shown (Neal and Hulme, 1958), that the addition of malate to peel disks of Bramley's Seedling apples which have passed the peak of their climacteric rise in respiration causes a sharp increase in CO_2-evolution. There is no corresponding increase in O_2-uptake. Malate-decarboxylation is associated with the onset of the respiration climacteric in the fruit and continues at a high rate after the climacteric peak is attained. From this earlier work it appeared that this decarboxylation, which is not confined to malate

although malate produces the greatest effect, might indeed be the major cause of the development of the respiration climacteric, namely the first manifestation of senescence. It was suggested that the small net increase in protein which is always associated with the climacteric (Hulme, 1937, 1954) might reflect a change in the enzyme pattern at this critical stage in the ontogeny of the fruit.

As part of a general study of senescence in plant organs, an examination was commenced of the effect of adding malate and certain other related acids to the peel of another variety of apple, namely, Cox's Orange Pippin. Once again, in post-climacteric fruits vigorous decarboxylation of malate (the 'malate effect') occurred, while in pre-climacteric fruit this malate effect was very small. Before proceeding to a more detailed physiological and biochemical investigation of the mechanism of the 'malate effect', it was considered advisable to see if the effect was confined entirely to the climacteric and post-climacteric phase in the life of the fruit. The present communication concerns the effect on the O_2-uptake and CO_2-output of adding malate to peel disks from Cox's Orange Pippin apples immediately after the fruit is detached from the tree and after periods of storage at 12° C. This was done at intervals from shortly after petal fall to just beyond the point at which the climacteric occurred in fruit on the tree. The 1959 growing season was particularly suited for such a study since favourable climatic conditions (particularly the absence of storms in September and early October) permitted a reasonable proportion of the fruit to remain on the tree until the climacteric peak was attained. This is an unusual occurrence.

By duplicating the experiments with fruit from two series of trees grown in the same orchard but having different manurial treatments, it was hoped to obtain information on the variability of the responses to the addition of organic acids to the peel tissue. Some data was also obtained for apples grown on rootstock M. IX, which, in contrast to the vigorous rootstock M. XII used in the main experiment, is a dwarfing stock. 'Petal fall' was, in all three cases, 10 May 1959.

MATERIAL AND METHODS

Sampling technique. Apples from 29-year-old Cox's Orange Pippin trees in a manurial trial at East Malling Research Station were used. Two treatments were chosen as giving, in general, 'good-' and 'bad'-keeping apples. Fruits from four trees in each treatment were used. All trees received phosphate and potash, but differed in that one set was cultivated and received nitrogen (NPK—the 'bad' keepers). The other set received no nitrogen and was grassed down in 1953 (PKS—the 'good' keepers). All these trees were on rootstock M. XII and the general layout of the plots and the amounts of fertilizer applied have been described by Wilkinson (1957). The results for Cox's Orange Pippin apples grown on rootstock M. IX were obtained from samples prepared from 25 trees grown on this stock in a different orchard at East Malling Research Station (series M2).

TABLE I

Details of the fruit of Series NPK and PKS

	Series NPK						Series PKS					
Pick	Date (1959)	Days from petal fall	Days in store	Total wt. (g.)	No. of fruits	Av. wt. (g. per fruit)	Date (1959)	Days from petal fall	Days in store	Total wt. (g.)	No. of fruits	Av. wt. (g. per fruit)
1a	3 June	24	0	97·0	32	3·0	5 June	26	0	138	32	4·3
b			15	94·0	32	2·9			13	139	32	4·4
c			34*	96·5	32	3·0			28*	133	32	4·2
d			41*	96·0	32	3·0			39*	141	32	4·4
2a	21 July	72	0	1,130	20	56·5	23 July	74	0	1,030	20	51·5
b			14	1,172	20	58·6			13	1,020	20	51·0
c			31	1,144	20	57·2			29	1,062	20	53·1
d			45	1,127	20	56·4			46	1,043	20	52·2
3a	18 Aug.	100	0	2,027	20	101·3	20 Aug.	102	0	1,791	20	89·6
b			9	2,093	20	104·7			8	1,856	20	92·8
c			24	1,980	20	99·0			21	1,869	20	93·5
d			38	2,211	20	110·6			36	1,781	20	89·1
4a	1 Sept.	114	0	2,351	20	117·6	3 Sept.	116	0	1,936	20	96·8
b			8	2,419	20	121·0			8	1,948	20	97·4
c			29	2,433	20	121·7			19	1,933	20	96·7
d			41	2,549	20	127·5			40	1,899	20	95·0
5a	15 Sept.	129	0	2,567	20	128·4	17 Sept.	131	0	2,185	20	109·3
b			6	2,619	20	131·0			7	2,302	20	115·1
c			17	2,790	20	139·5			18	2,156	20	107·8
d			30	2,806	20	140·3			29	2,174	20	108·7
6a	21 Sept.	135	0	2,847	20	142·4	23 Sept.	137	0	2,373	20	118·7
b			7	2,839	20	142·0			6	2,434	20	121·7
c			17	2,811	20	140·6			16	2,392	20	119·6
d			35	2,893	20	144·7			35	2,257	20	112·9
7a	23 Oct.	167	0	2,109	15	140·6	21 Oct.	165	0	1,642	—	117·3
b			21	1,639*	12†	136·6			21	1,722	—	108·0

* In these samples one or two rotted fruits were discarded. The average weight was not seriously affected.
† Total fruits remaining on trees.

In the series NPK and PKS the four samples were prepared at each picking as follows: each sample was made up of an equal number of apples, picked at random, from each of the four trees. One sample (the initial) was placed at 1° C. for the immediate preparation of the peel tissue, whilst the other three samples (storage samples) were each placed in a desiccator of a respiration train (Hulme, 1938) at 12° C. The respiration rates of the whole fruit given later are the CO_2-outputs of these samples of fruit. The size of the samples varied from 32 fruits in the first pick where the fruits were small, to 15 fruits at the final pick. In series M2 at least one apple per tree was taken for each sample. The decision when to take a sample out of storage for study was based, in general, on the rate of respiration of the sample, especially in relation to the climacteric rise. Details of the fruit of series NPK and PKS are shown in Table I and, for series M2, in Table II.

TABLE II

Details of the fruit of Series M2

Pick	Date (1959)	Days from petal fall	Av. weight (g. per fruit)
1	26 May	16	1·23
2	2 June	23	2·24
3	4 June	25	3·33
4	9 June	30	5·66
5	11 June	32	5·75
6	23 June	44	12·2
7	25 June	46	15·1
8	30 June	51	20·3
9	10 July	61	30·0
10	16 July	67	47·5
11	28 July	79	63·1
12	30 July	81	69·8
13	7 Aug.	89	79·9
14	11 Aug.	93	95·6
15	13 Aug.	95	95·1
16	8 Sept.	121	130·7
17	24 Sept.	137	147·1
18	6 Oct.	149	137·2
19	14 Oct.	157	154·0

Preparation of tissue slices. For most of the work disks of peel were prepared at 1° C. as previously described (Neal and Hulme, 1958). This method is satisfactory with relatively large apples (20 g. and larger) where the prepared 'peel' consists of epidermis, hypodermis, and a layer of cortical tissue two or three cells thick and free from intercellular spaces (see Fig. 1). With smaller apples the subepidermal tissue appears more closely knit with the cortical tissue and attempts to prepare 'peel' by the usual method resulted in a general fragmentation of the peel as it was scraped to remove cortical tissue. With small apples (all of pick 1, NPK and PKS, and the first 10 picks of M2 the results for which are shown as solid circles in the figures), therefore, the 'peel' tissue was obtained by cutting from the outside of the fruit with a sharp

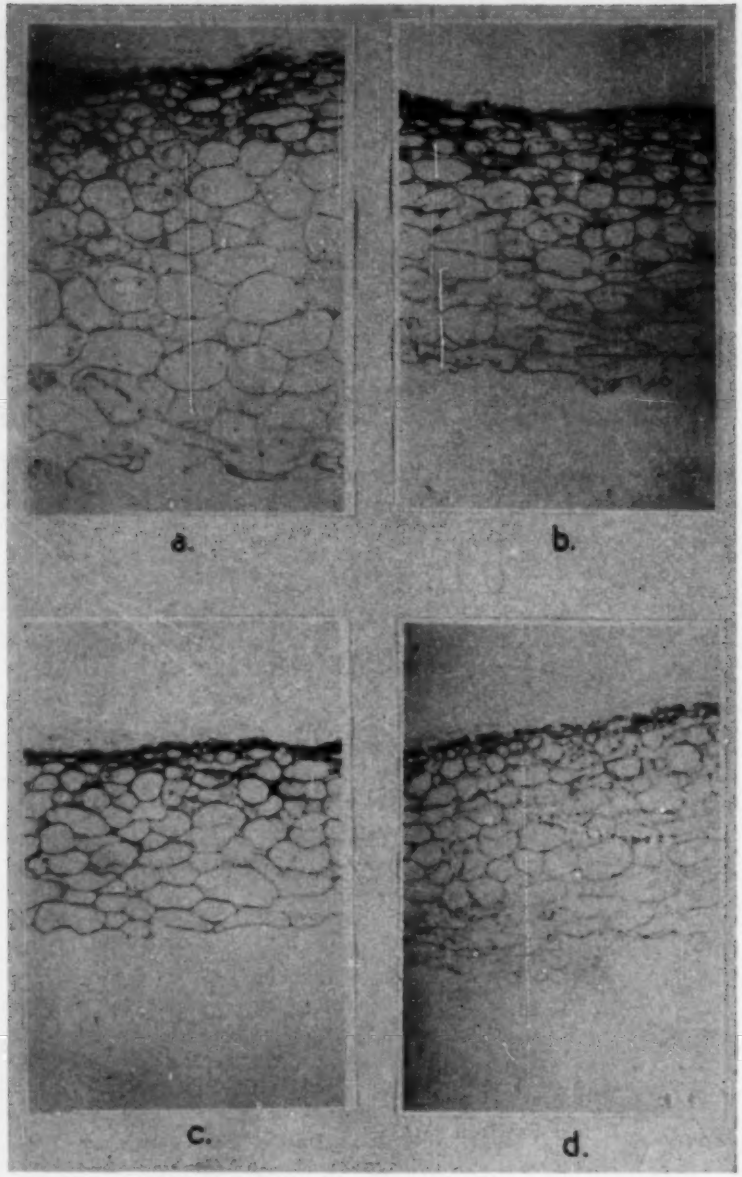


FIG. 1. Photomicrographs of peel disks and peel slices of Cox's Orange Pippin apples used in the Warburg experiments—

a. Pick 3a disk material before final scraping with porcelain spatula (see Neal and Hulme, 1958).
b. Pick 3a disk material after final scraping, i.e. as used in Warburg respirometer.
c. Pick 7b, slice cut with sharp scalpel.
d. Pick 7b, disk material as used in Warburg respirometer.

scalpel strips as thin and as uniform as possible. Fig. 1 illustrates the difference in the structure of the peel obtained by the two methods.

The difference in certain physiological properties of the different examples of 'peel' will be discussed later. It should be emphasized here, however, that, as earlier work (Neal and Hulme, 1958) has also shown, disks of peel prepared by the standard method are comprised of similar tissue over the major part of the period in the life of the fruit here examined.

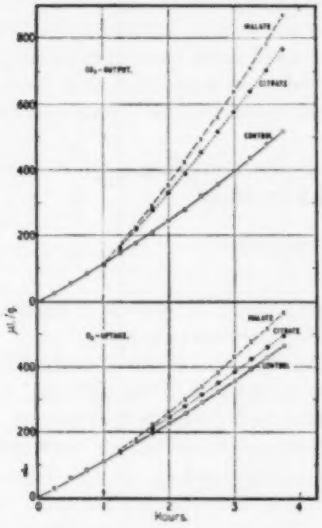


FIG. 2

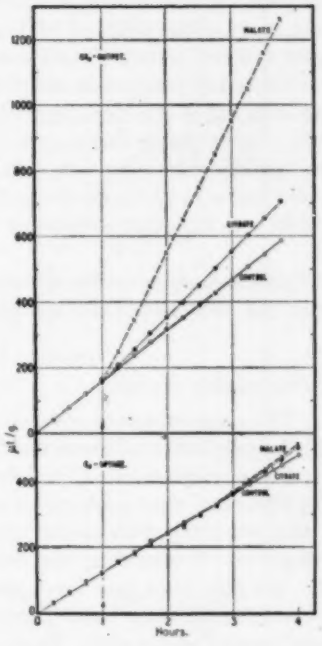


FIG. 3

FIG. 2. O_2-uptake and CO_2-output of peel slices. Series PKS, pick 1b (see Table I). Malate or citrate added after 1 hour (point marked with an arrow on base lines).

FIG. 3. O_2-uptake and CO_2-output of peel disks. Series NPK, pick 4a. Malate or citrate added after 1 hour (point marked with an arrow on base lines).

Manometric technique. The arrangements of the contents of the flasks of the Warburg respirometer and the method of using this apparatus were as described by Neal and Hulme (1958); both O_2-uptake and CO_2-output were determined. A lower temperature (20° C.) was used in the present experiments to be nearer the temperature of the orchard and of storage of the whole fruit. The peel was placed in phosphate buffer (pH 4·0) in the 1° C. room as it was being prepared. When the amount required for a working run was ready, it was washed twice in cold buffer, dried between large sheets of filter-paper, weighed, and immediately transferred to the Warburg flasks (at 1° C.).

Bubbling overnight in buffer (Neal and Hulme, 1958) was not necessary with the present material since, using the above technique, the basic respiration rate remained constant or fell only very slightly during a 4-hour or 5-hour run in the respirometer. This will be clear from Figs. 2 and 3.

Addition of acids. The acids, after adjustment to pH 4 with KOH, were added at the appropriate time from the side arms of the Warburg flasks to give a final concentration of 0·1 M. This concentration gave, in general, the maximum increase in CO_2 production from malate. Although the main object was a study of the effect of adding malate ('malate effect') to respiring disks, citrate was also tested. Citrate was chosen in the present instance because, in spite of its key position in the Krebs cycle, it has previously been found to have no effect on the respiration of peel from mature apples (Neal and Hulme, 1958). Laties (1959) has suggested that citrate cannot penetrate the intact mitochondrion. If this is why it fails to affect peel-tissue respiration, it should be ineffective at all stages of development, unless it is assumed that the permeability of the outer membrane of the mitochondrion undergoes modification.

Chemical analyses of the tissue at the end of certain Warburg 'runs' were made; the results obtained will appear in a later paper.

EXPERIMENTAL RESULTS

A. Presentation of results

1. *The respiration rate of whole fruits* is expressed, for ease of comparison with the respiration of tissue disks, as μl. CO_2 per g. per hr. $\times$ 10.

2. *The respiration rate of peel disks.* Half an hour was allowed for the Warburg flasks and their contents to attain the temperature of the bath (20° C.). At this point the manometer taps were shut and readings taken every 15 minutes; the first reading was always ignored and the additions were made from the side arms one hour subsequent to this reading. In the 'control' experiments distilled water (brought to pH 4 with phosphoric acid) was added instead of the acids. Because of the difficulty of adding malate at the high concentrations required to give a final concentration of 0·1 M., the Warburg flasks were out of the bath for a considerable portion of the first 15-minute interval. Hence, the volume change during this first 15-minute interval was of little significance and was ignored and is not shown on the graphs. During the next 15-minute interval, penetration of the malate (or citrate) to the active centres was probably not complete (penetration of 'apparent free space'—see Robertson, 1959), and although the gas exchange for this period is shown in Figs. 2 and 3 it was ignored in the calculations.

In the calculation of the basic respiration rate (O_2-uptake and CO_2-output) of the peel disks, the total uptake or output for the first 3½ hours (excluding the first 30 minutes after additions of water from the side arms) was divided by three, the results being expressed as μl. gas per g. per hour. From Fig. 2 it will be seen that the rate of O_2-uptake and CO_2-output increases somewhat after 2½ hours and 1½ hours respectively. The figures calculated as above for

the average uptake and output would, in this case, be slightly higher than the true initial rate. Such a change in rate over the first 3 hours in the Warburg respirometer is, however, exceptional and is, no doubt, due to the imminent change in respiration rate of the whole-apples of this sample from a falling one to a rising one. (See Fig. 6.)

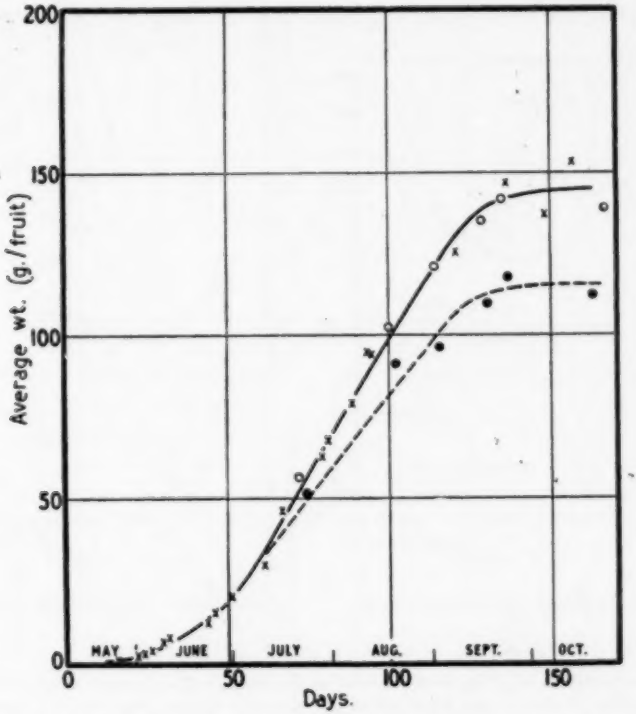


FIG. 4. Growth curves for Cox's Orange Pippin apples, 1959. ○ = series NPK; ● = series PKS; × = series M2.

The values of the malate effect and citrate effect (both O_2-uptake and CO_2-output) represent the differences between the gaseous exchange in the malate (or citrate) sample and that of the control sample, expressed as μl. gas per g. per hour, over the period from 30 minutes ('penetration' period; see above) after the addition of the acid salt to 2½ hours after that addition.

B. Changes occurring in fruit attached to the tree

1. *General.* The growth curves for the fruit of the three series are shown in Fig. 4. For the first 60–70 days from petal fall, the rate of growth is remarkably similar for all three series. Thereafter, although there are few results available for series NPK in the middle period of growth, the rate of growth of NPK

and M2 are very similar. These results, taken as a whole, suggest that there are three phases in the growth of all three series of fruit. In the early phase ending about 60 days after petal fall, growth-rate is increasing; a steady rate of growth follows until about 120–30 days, after which increase in weight of the fruit practically ceases. A similar course of events has been frequently

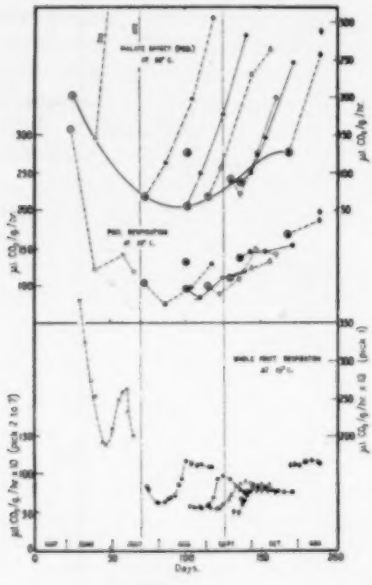


FIG. 5

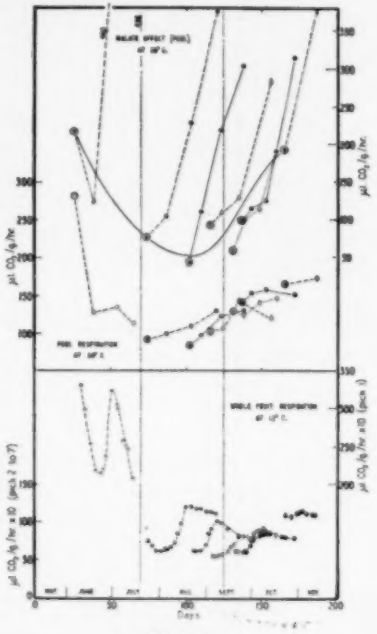


FIG. 6

FIG. 5. Respiration rate (CO_2 production) of whole apples, CO_2-output of peel disks (or slices —all samples of pick 1), and the effect of adding malate on the CO_2-output of peel, immediately on picking (initial samples) and after storage at 12° C. The points for the initial samples of peel are 'ringed' for clarity. The solid circles at 100 days and 178 days refer to *slices* for comparison with disks from the same fruit. Series NPK.

FIG. 6. As for Fig. 5, series PKS. All results for peel of pick 1 refer to slices; all other results are for disks.

reported for apples (notably Archbold, 1932); the length of each of the three phases of development varies with the variety of fruit and, to a less extent, with the climatic conditions.

Figs. 5 and 6 show the respiration rate (CO_2-output) of the whole fruit, of the peel tissue, and of the effect of adding malate on the CO_2-output of the peel tissue, for series NPK and PKS. These figures also show the sequence of events in the fruit detached and stored at 12° C.; these will be discussed later. Results for the series M2 fruit are shown in Fig. 7. There are no results available for the respiration of the whole fruit in this series, but included in

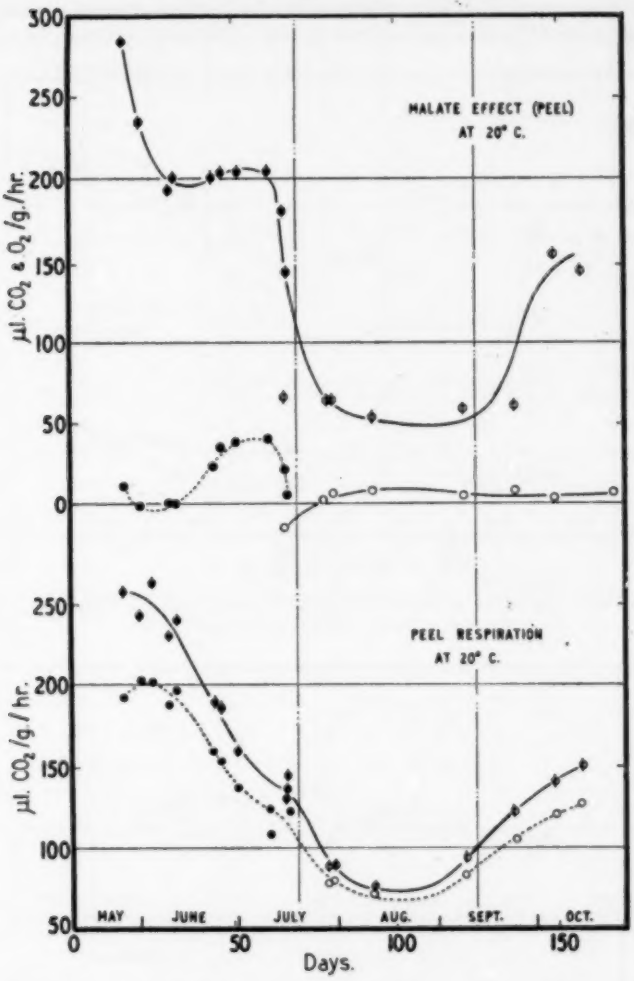


FIG. 7. Respiration rate and effect on respiration of adding malate, peel tissue series M2.
● = O_2-uptake of slices; ○ = O_2-uptake of disks;
◖ = CO_2-output of slices; ◑ = CO_2-output of disks.

the figures are the basic O_2-uptakes of the peel and the O_2-uptakes due to added malate. The corresponding results for O_2-uptake for series NPK and PKS are shown in Figs. 8 and 9.

Results for the addition of citrate are shown in Fig. 10. It will be seen that, for series M2, results are less numerous than for the effect of added malate. There are no data for fruit in storage.

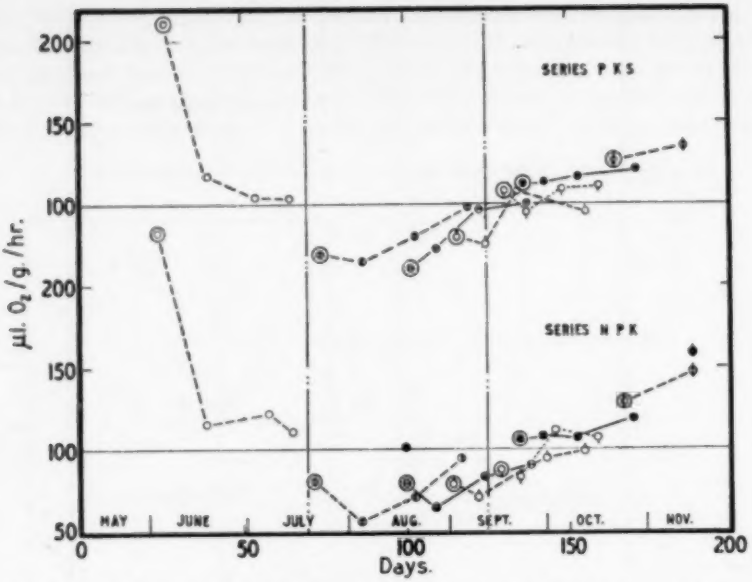


FIG. 8. 'Basic' O_2-uptake of peel (slices or disks) corresponding to CO_2-output shown in Figs. 5 (series NPK) and 6 (series PKS).

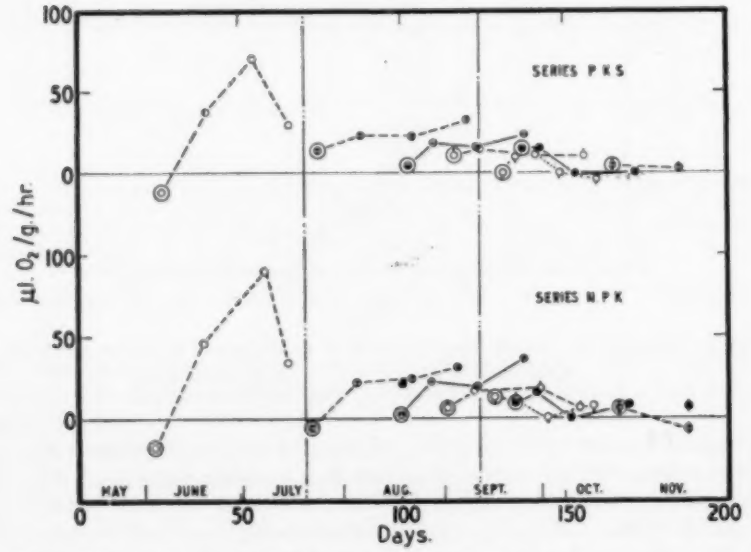


FIG. 9. Effect on O_2-uptake of adding malate to peel. These results correspond with the results for CO_2-outputs shown in Figs. 5, 6, and 7.

For clarity, the results for the initial samples of fruit from series NPK and PKS are surrounded by a circle, except for the respiration of whole fruits.

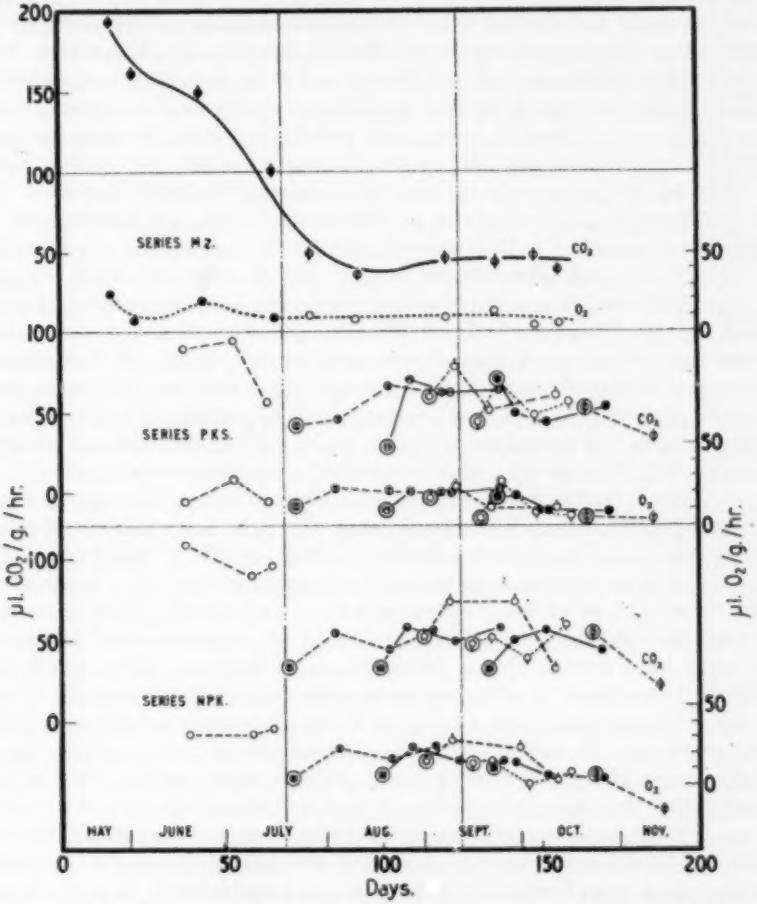


FIG. 10. The effect of adding citrate to peel (slices, solid circles, and disks, open circles) on O_2-uptake and CO_2-output. Series NPK and PKS results correspond with those for the malate effect given in Figs. 5, 6, and 9, except that there are no 'citrate' effects for the initial samples of pick 1. Results for series M2 correspond with results shown in Fig. 7, except that the citrate effect was not measured on all samples. The symbols are the same as described for earlier figures.

2. *Basic respiration of whole fruit and peel tissue.* (See Figs. 5, 6, 7, and 8.) The CO_2-output of the whole fruits follows a familiar pattern (Kidd and West, 1945; Hulme, 1954). The high respiration rate of the young fruit falls rapidly to a relatively steady rate reached about 70 days from petal fall.

Finally, the respiration climacteric commences at about 120–30 days from petal fall. In the peel tissue both the CO_2-output and the O_2-uptake follow the same general pattern as that of the CO_2-output of the whole fruit except that, as might be expected from the higher respiration rate of peel than of pulp tissue (Neal and Hulme, 1958), the CO_2-output is at a higher rate. The check which intervenes, between days 30 and 60 in series M2, in the general fall in respiration during the first 80–90 days, is quite usual in apples. It has, no doubt, been missed in series NPK and PKS because no readings were taken between 20–30 days and 70–80 days after petal fall.

The picture in the early stages (pick 1, series NPK and PKS, picks 1–10, series M2—the points marked with solid circles in Fig. 7) is complicated by the use of tissue *slices*. It is possible from the present data to compare the respiration of such slices with the normal disks in only a few cases, namely pick 2, NPK, and storage samples from NPK, 188 days after petal fall (Fig. 5) and pick 10, M2 (Fig. 7). These few samples, in which 'slices' and 'disks' were carefully prepared from the same apples for purposes of comparison, show that in the early stages of development there is an appreciable divergence in CO_2-output and, to a lesser extent, in the O_2-uptake of the two tissue preparations. The more densely packed, less vacuolated outer cells are, clearly, more active. This matter will be referred to again later.

3. *Effect of adding malate to peel tissue.* The effect on CO_2-output is clear from the upper curves ('circled' points) in Figs. 5, 6, and 7 and the effect on O_2-uptake from Fig. 7 (M2) and Fig. 9 (NPK and PKS). The form of the CO_2-output curve follows the same general pattern as the basic respiration of the tissue with series M2, illustrating a more emphasized 'plateau' between 40 and 60 days from petal fall, than for basic CO_2-output. In fact, the results for fruit 'on the tree' support the suggestion of Neal and Hulme (1958) for detached Bramley's Seedling apples that the malate effect develops during the respiration climacteric. Change in O_2-uptake does not, however, follow the pattern of CO_2-output and, apart from the peak at 50–60 days from petal fall in series M2, shows relatively little effect of adding malate. This, again, agrees with the general conclusions of Neal and Hulme.

4. *The effect of the addition of citrate.* There are no data available on the effect on the O_2-uptake and CO_2-output of adding citrate to slices as compared with disks of peel. Presumably, where there is a citrate effect, as with malate, slices will give a greater response than disks. In Fig. 10, this difference would affect the first pick of series NPK and PKS and the first four picks shown (not all the picks of this series were tested for citrate effect) for series M2. Bearing this factor in mind, the general trend of the citrate effect on CO_2-output is similar qualitatively to the malate effect, although quantitatively it is considerably smaller, during the first and possibly during the second stage of development. There is, however, very little effect of adding citrate during the development of the respiration climacteric. The addition of citrate has very little effect on O_2-uptake at any time during development with the possible exception of the very early stages (series M2, Fig. 10) where the curve

of O_2-uptake shows a similarity, on a much reduced scale, to that for addition of malate (Fig. 7).

C. Changes occurring in detached fruits

1. *Basic respiration of whole fruit and peel tissue.* (See Figs. 5 and 6.) It would appear that the series of curves shown here are the first published data for the respiration at normal (as opposed to cold-storage) temperatures of English-grown Cox's Orange Pippin apples picked at sequential stages of development. With the first pick it was not possible to carry the fruit through to the period where a climacteric might develop because, unlike Bramley's Seedling apples, the Cox's Orange Pippin is particularly susceptible to fungal attack. Even at the end of the 41 days in storage (pick 1) several fruits had become rotten (see Table I). The peak which followed rapidly on the initial fall is not the normal climacteric associated with 'ripening' (see Hulme, 1954, for a similar series of respiration curves for Bramley's Seedling apples). This early peak, sometimes merely a plateau in a general fall, is always found with very young fruit of most varieties. For Bramley's Seedling apples, although not shown in the published curves of Hulme (1954), it was indeed observed; typical examples may be seen in Figs. 3 and 5 of a paper by Kidd and West (1945).

The most interesting point about the present series of curves is that in picks 2, 3, 4, and 5, where the climacteric had not, or had only just (pick 5), set in on the tree, the rate of CO_2-production at the climacteric peak is progressively lower. In Bramley's Seedling apples the value of CO_2-production at the climacteric peak *rises* progressively as the fruit matures (Kidd and West, 1945; Hulme, 1954).

As with fruit attached to the tree (i.e. the initial samples), the general trend of the respiration of the peel in relation to the onset of the climacteric follows that of the whole fruit. However, two striking differences appear between the CO_2-output of the whole fruit and that of the peel. Firstly, the climacteric peak is reached more slowly in the peel tissue—indeed the results do not proceed as far as the peak in some picks. Secondly, in spite of this incompleteness in the results for the peel, it seems fairly certain that, in contrast with the whole fruit, the value of the CO_2 production at the peak increases in the peel as the date of picking gets later. The peel, in fact, behaves like whole Bramley's Seedling apples. The O_2-uptake of the peel tissue (Fig. 8) in general follows the trend of CO_2-output although the R.Q. is always between 1·2 and 1·3.

As regards the difference between slices and disks, presumably, the same strictures apply as for the results for the 'on the tree' samples (p. 321). As the fruit becomes more mature (Fig. 5 and Fig. 8, last pick, storage sample 188 days from petal fall), the differences in the results for slices and disks become less.

Again the results as a whole fall into a pattern of three stages, the first stage being up to about 70 days from petal fall and the second stage lasting until the onset of the climacteric (120–30 days).

2. *The effect of adding malate to the peel tissue.* Before considering the results obtained with the present series of fruit, it is relevant to mention some data

obtained in the 1958 season with fruit from the trees of series NPK and PKS picked in late August and in October and stored for short periods. These results, which are presented in Table III, show that in such fruit, like the Bramley's Seedling apples used by Neal and Hulme (1958), a malate effect develops in the peel during the progress of the respiration climacteric in the whole fruit.

TABLE III

Respiration rate of whole apples and respiration rate and malate effect in peel tissue prepared therefrom (1958 season)

Date (1958)	Series	Whole fruit respiration $\mu l./CO_2/g.$ (12° C.)	Tissue disk respiration $\mu l./g.$ (20° C.)		Malate effect $\mu l./g.$ (20° C.)		Remarks
			O_2-uptake	CO_2-output	O_2-uptake	CO_2-output	
26 Aug.	NPK	7·0	98	124	16	87	On tree; pre-climacteric.
16 Oct.	NPK	10·0	110	148	13	114	On tree; climacteric developing.
28 Nov.	NPK	—	123	249	36	354	Cold stored fruit; see test.
28 Aug.	PKS	6·5	88	102	15	77	On tree; pre-climacteric.
23 Oct.	PKS	9·2	116	142	17	123	On tree; climacteric developing.
23 Sept.	PKS	9·6	84	109	31	248	In store at 12° C. since 28 Aug. Whole fruit at climacteric peak.
5 Dec.	PKS	—	116	139	30	233	Cold stored fruit; see test.

In the table, 'cold stored' fruit refers to fruit stored at 4° C. until 3 days before the Warburg experiments were performed. During these 3 days the fruit was held at 12° C. for the climacteric to develop (see Neal and Hulme, 1958). It will be seen from the table that when once the malate effect has commenced it continues to increase considerably after the climacteric peak in the whole fruit.

The results for 1959 confirm that in *maturing* fruit (Figs. 5 and 6, 120–30 days from petal fall) the onset of the malate effect accompanies the onset of the respiration climacteric. During stages 1 and 2 the picture is, however, quite different. In stage 1 the malate effect follows, qualitatively, the general pattern of change in the basic respiration of the tissue, although the peak at about 60 days from petal fall is not a true climacteric. During stage 2 (70–125 days) the results conflict sharply with the concept of a relationship between the onset of the climacteric and the development of the malate effect. Generally, during this stage, the addition of malate causes a large outburst of CO_2 production immediately after picking and 10–20 days before the onset of the respiration climacteric (picks 2, 3, and 4, series NPK and PKS). There is a suggestion, however, that as this stage merges into stage 3 when the climacteric is imminent, the rise in basic CO_2-output and the increase in the malate effect are occurring at about the same time (series PKS, pick 4).

In the detached fruit, the effect of adding malate on O_2-uptake (Fig. 9) shows no similarity to the effect on CO_2-output. This divergence is particularly striking in pick 1 during the first 15 days in storage. This general decarboxylation of malate unaccompanied by any very marked increase in

O_2-uptake suggests—nothing more—that the malate effect is essentially the same process throughout the life of the apple fruit.

3. *The effect of the addition of citrate.* It will be seen from Fig. 10 that it is only during stage 1 that the addition of citrate to the peel tissue shows a response commensurate with that of adding malate. Even during this stage (pick 1), however, the trend of events for each acid does not run parallel. If indeed the lack of effect of citrate (in mature apples) is due to lack of penetration of the mitochondrion (see p. 322) then the outer membrane of the mitochondrion must be different in very young as compared with mature fruit. Throughout all three stages of development the addition of citrate has very little effect on the O_2-uptake.

DISCUSSION

The present results show that the overall picture is remarkably similar for all three series of samples, especially when rapid changes are in progress. Taking into account the differences in cultural conditions and rootstocks, it would appear that not only are the trends in respiration of the fruit during growth fundamental but that the respiration (O_2-uptake and CO_2-output) of the peel tissue, and its responses to the addition of malate and citrate, are also fundamental.

Owing to the favourable climactic conditions, the present series of results illustrate particularly well the development of the climacteric on the tree, both in the whole fruit and the separated peel. Changes in rate of growth, and rate of respiration of the whole fruit and of the peel tissue, can be divided broadly into three stages. These stages—from petal fall to 60 days, from 60 days to 125 days, and from 125 days onward—are indicated by vertical interrupted lines in the figures. There is nothing new in this: three similar stages of development covering approximately the same periods in time, were apparent in respiration rates and in the balance between protein and non-protein nitrogen in the results for Bramley's Seedling apples (Hulme, 1937 and 1954). In the results for 1954, during the second stage, total nitrogen remained fairly constant (after a rapid fall in stage 1) and protein nitrogen remained a constant proportion of the total. In fruit detached and stored at 12° C. during this stage there was very little change in the balance between protein and non-protein nitrogen at least during the first 10 days (Hulme, 1937).

The present paper provides new data on metabolic changes during these three stages and it is proposed to discuss this data in terms of the three stages of development. Although the present results centre round the metabolism of the aliphatic organic acids, no attempt will be made at this stage to analyse them strictly in terms of the Krebs cycle. The present position of our knowledge of the organic acid metabolism of apples in relation to the Krebs Citric Acid Cycle will be discussed elsewhere (Hulme and Flood, 1960).

In stage 1 there is considerable evidence that the metabolic pattern in the peel tissue is different from that of following stages. The O_2-uptake is considerably influenced by the addition of malate and of citrate; uniquely

CO_2- output shows a marked response to the addition of citrate. Unpublished results also indicate that inhibition by malonate of the basic respiration and of the malate effect is greater during this stage than subsequently. This special status of stage 1 may be due, in part, to the fact that active cell division continues at a considerable rate in the apple fruit up to about 30 days from petal fall (Tukey and Young, 1942; Smith, 1950). According to Tukey and Young (1942), in American varieties of apples cell expansion continues for about 60 days from full bloom, after which the cell walls commence to thicken. The acid metabolism of this stage of development would repay a more detailed study.

During the second and third stages, respiration of the peel tissue follows the expected course with a period of steady or slightly falling rate followed by a rise to the climacteric. The earlier onset of the climacteric and the increasing peak value, as compared with a falling one in the whole fruit, cannot be explained at present other than by postulating a looser 'control' in the separated tissue as compared with the whole fruit.

An important point which needs careful consideration is the unexpectedly rapid and immediate development of the malate effect as soon as the fruit is detached from the tree during the second stage of development. This problem is also associated with the first stage since the peak in basic respiration here is not a true climacteric. Until more data are available it is impossible to do more than speculate as to the cause of this 'uncoupling' of the malate effect from the climacteric in the first two stages of development. The fall in the magnitude of the malate effect immediately after picking, in pick 1 (Figs. 5 and 6), is unlikely to be due to a shortage of enzyme for two reasons, (1) the protein content of the fruit is at a maximum here (Hulme, 1954) and (2) there is a rapid reversal to a large increase in malate effect after 24–26 days in storage while, by analogy with results for Bramley's Seedling apples (Hulme, 1954), protein is falling. It cannot be due to a lack of direct substrate (i.e. malate or a closely associated acid utilized in basic respiration) because of the initial fall in the malate effect (1st pick, 1st storage sample). It is, however, probably significant that in the first storage samples of pick 1, basic O_2-uptake is falling rapidly and this fall is strikingly reversed on the addition of malate (Figs. 8 and 9). Since citrate is 'active' at this stage (Fig. 10), possibly there is a shortage of some respiratory substrate far removed from malate but, nevertheless, ultimately involved in the Krebs cycle—for example, pyruvate or acetyl Co-A which feeds into the cycle. We have found that the tissue at this stage is particularly susceptible to inhibition by iodoacetate (to which triose phosphate dehydrogenase is particularly susceptible); the higher susceptibility to malonate of this young tissue, as compared with that from more mature apples, has already been mentioned. The development of a vigorous malate effect in the longer-stored samples of pick 1 may be due to more of this substrate (e.g. the pyruvate mentioned above) becoming available during the processes connected with the increased basic respiration of the fruit after 15–16 days in store (Figs. 5 and 6).

In stage 2 the low malate effect initially cannot be due to shortage of enzyme

since the malate effect commences to rise immediately after picking; by analogy with the Bramley's Seedling data (Hulme, 1954) protein-content remains fairly steady after picking. Again, it is possible that a 'remote' substrate was limiting in the initial sample. Direct malate-substrate cannot be limiting because of the low initial malate effect. In stage 3, as in all the other results for fruit approaching maturity, the development of the malate effect follows the onset of the respiration climacteric.

An alternative explanation of the lack of correspondence between malate effect and onset of the climacteric in stages 1 and 2, might be that an inhibitor of the malate effect continually enters the fruit from the tree. If this inhibitor is present in large amounts during the early stages, and decreases as the fruit enters stage 2, then enough may remain in the fruit during stage 1 to inhibit the malate effect during the first storage period. In stage 2 this hypothetical inhibitor disappears rapidly, so that the malate effect develops almost immediately after picking.

Whatever the 'controlling influence' of the tree, it is possible to argue that, on detachment, the apple initially tends to imitate the rate of the malate effect as expressed by the curve for the effect 'on the tree'. For example, in stage 2, when the rate of change of the malate effect on the tree is almost zero, the malate effect is quite uncontrolled after the fruit is detached. Until more is known of the mechanism of the malate effect and of the site of the relatively large amount of the malate present in the apple itself (presumably there are at least two sites, an 'active' one in the cytoplasm and an inactive pool in the vacuole), it is profitless to speculate further on how control is exercised on the decarboxylation of added malate.

One further point of interest: the present results indicate that the malate effect is most active in the epidermis and hypodermis since, in the outer layers of the peel, decarboxylation of malate is relatively much greater than would be suggested by the difference in the basic respiration between slices and disks (Figs. 5 and 7). This is in agreement with the findings of Neal (1957) that the peel of Bramley's Seedlings exhibits a much greater malate effect than does the pulp (cortex).

Having established the broad picture of malate metabolism in the apple, it is proposed to examine the mechanisms concerned by a study of the enzyme systems, both particulate and soluble, involved in the transformation of malate by apple tissue. It is hoped to link this work with morphological changes in the peel, using both the light and the electron microscope.

ACKNOWLEDGEMENTS

We wish to thank Dr. A. B. Beakbane of East Malling Research Station for the preparation of the sections given in Fig. 1, and Mr. A. J. Topping of Ditton Laboratory for taking the photographs of the sections.

(*The work embodied in this paper forms part of a research programme of the Agricultural Research Council*)

LITERATURE CITED

ARCHBOLD, H. K. (1932). Chemical studies in the physiology of apples. XII. Ripening processes in the apple. *Ann. Bot.* **46**, 407.

HULME, A. C. (1937). The metabolism of nitrogen of apple fruits. *Rep. Food Investigation Board* for 1936, p. 126.

—— (1938). An apparatus for measuring the output of carbon dioxide by a sample of 2–4 kg. of apple fruits. *Rep. Food Investigation Board* for 1937, p. 133.

—— (1954). Studies in the nitrogen metabolism of apple fruits. The climacteric rise in respiration in relation to change in the equilibrium between protein synthesis and breakdown. *J. Exp. Bot.* **5**, 159.

—— and FLOOD, A. E. (1960). Evidence for the participation of the Krebs Cycle in apple fruits. In preparation.

KIDD, F., and WEST, C. (1945). Respiratory activity and duration of life of apples gathered at different stages of development and subsequently maintained at a constant temperature. *Plant Physiol.* **20**, 467.

LATIES, G. C. (1959). The organisation of energetic systems. Proceedings of Section 11, Physiology, IXth International Botanical Congress, Montreal, 1959.

NEAL, G. E. (1957). *Some manometric and biochemical studies of the organic acid metabolism of Bramley's Seedling apples.* Ph.D. Thesis, Univ. of London.

—— and HULME, A. C. (1958). The organic acid metabolism of Bramley's Seedling apple peel. *J. Exp. Bot.* **9**, 142.

ROBERTSON, D. N. (1959). Organised discussion on apparent free space. IXth International Botanical Congress, Montreal, 1959.

SMITH, W. H. (1950). Cell multiplication and cell enlargement in the development of the flesh of the apple. *Ann. Bot.* (N.S.) **14**, 23.

TUKEY, H. B., and YOUNG, O. J. (1942). Gross morphology and histology of developing fruit of the apple. *Bot. Gazette*, **104**, 3.

WILKINSON, B. G. (1957). The effect of orchard factors on the chemical composition of apples. 1. Some effects of manurial treatment and of grass. *J. hort. Sci.* **32**, 74.

Starch Dissolution in Tobacco Leaves in the Light

TSIN-TZE CHAN AND I. F. BIRD

A.R.C. Unit of Plant Physiology, Imperial College of Science and Technology, London, S.W.7

Received 2 April 1960

SUMMARY

Single attached leaves of tobacco, depleted of starch, were allowed to assimilate a fixed amount of $^{14}CO_2$. About 50 per cent. of the ^{14}Carbon assimilated was translocated from the lamina concurrently with assimilation. Of that remaining in the lamina 80 per cent. was incorporated into starch and sugar. Plants were then placed in air and continuous light of different intensities as a means of controlling further starch synthesis. At about 500 f.c. there was no change in amount of starch after 72 hours, but half the radioactivity was lost. At light intensities lower than 500 f.c. percentage activity lost was greater than the percentage amount lost. In sunlight, where new starch synthesis was very rapid, loss in activity from the radioactive portion was prevented. In all circumstances activity virtually disappeared from both hexose and sucrose. It is concluded that starch dissolution proceeded in light even when the amount was increasing. It is further suggested that the loss was mainly from outer layers of the starch grains which were replaced by newly synthesized material. Loss of sugar was by direct translocation rather than passage to starch, and complete replacement by new photosynthate occurred in 24 hours.

INTRODUCTION

In an investigation of translocation from a single attached tobacco leaf after a short period of assimilation of $^{14}CO_2$, Jones, Martin, and Porter (1959) found that, in continuous light, the sugar content of the leaf lamina remained virtually constant while the amount of starch approximately doubled. The concomitant changes in radioactivity of these substances suggested that sugar was completely renewed in about 24 hours, and that about half the radioactive starch present immediately following assimilation of $^{14}CO_2$ was lost in the light after about 96 hours, although the total amount had increased. The observations, particularly with respect to starch, required confirmation because of the small number of samples examined.

A more extended study has now been made of the breakdown of starch in light, using different light intensities as a means of controlling the amount of starch present.

METHODS

Tobacco plants ('White Burley') were grown in a greenhouse in 10-in. pots. Leaves which had just reached full expansion, as determined by length measurements, were selected from those between the 13th and 23rd to emerge. The lamina of each selected leaf was trimmed to 175 sq. cm. using a template, and then enclosed in a perspex leaf chamber and supplied with 111·5 mg.

$^{14}CO_2$ in a closed circuit (Jones *et al.*, loc cit.). The specific activity of the CO_2 was in the range 166×10^3 to 174×10^3 c.p.m./mg. carbon in the several experiments.

The whole apparatus was set up in the greenhouse and illumination was by sunlight or by HPMV lamps (400 watts) placed about a foot from the leaf chamber. Light intensities were measured at the leaf surface with a photometer. Assimilation of all the $^{14}CO_2$ required times up to 3 hours. When it was complete the leaf chamber was removed and at once a sample of 10 leaf disks, 1 cm. diameter, was cut from the lamina with a cork-borer. The whole plant was then kept in air in continuous light for 72 or 24 hours. High light intensity was maintained by exposure to sunlight during the day and to two HPMV lamps at night. This gave a minimum intensity of 1,500 f.c. and in sunshine intensities of 3,000 f.c. or more. Medium and low light intensities were obtained by blocking out the daylight and altering the distance of the lamps from the leaf surface.

At the end of the light period a second sample of leaf disks was cut. All samples were transferred at once to boiling ethanol and subsequently the amounts and radioactivity of starch and sugar were estimated by methods already described (Porter, Martin, and Bird, 1959). Total radioactivity in the leaf disks was estimated as the sum of that in the ethanol-insoluble and ethanol-soluble fractions by combustion of suitable samples to $^{14}CO_2$ (Porter *et al.*, loc. cit.).

RESULTS AND DISCUSSION

The amount of starch and sugar in 175 sq. cm. of leaf lamina after assimilation of 111·5 mg. $^{14}CO_2$ (equivalent to 76 mg. of hexose) in five different leaves varied between 28 to 41 mg. of starch, and 18 to 22 mg. of sugar (sucrose+hexose), or 1·27 to 1·83 mg. of starch and 0·80 to 1·0 mg. of sugar in a sample of 10 1-cm. leaf disks. The radioactivity remaining in the lamina, at the end of the assimilation period, as determined by combustion of the leaf tissue to $^{14}CO_2$, together with the total activity in starch and sugar, for three leaves is shown in Table 1. With attached leaves under the conditions of these experiments about half the carbon assimilated was translocated from the lamina either into the large veins and mid-ribs or out of the leaf, concurrently with assimilation. Of the remaining half, 80 per cent. was incorporated into starch and sugar. Sugar formed a rather higher percentage of the ethanol-soluble fraction than did starch of the ethanol-insoluble. The products becoming radioactive other than starch and sugar were not further characterized. Since all the $^{14}CO_2$ supplied was taken up, possible isotope effects during synthesis are considered unimportant here.

The general procedure followed was to place plants in the dark for 48 to 60 hours to deplete them of starch, and then expose the fixed area of a single leaf to the same amount of $^{14}CO_2$. The plant was then placed in the chosen light intensity. On the following day a second plant was similarly treated to provide a duplicate result. Since the high light intensities used depended on

TABLE I

Distribution of radioactivity in three samples of Tobacco leaf lamina after assimilation of $^{14}CO_2$

(175 sq. cm. of leaf assimilated, in about 3 hours, 111·5 mg. $^{14}CO_2$, total activity 5·0 to $5{\cdot}3 \times 10^6$ c.p.m.)

	% of total activity assimilated					
	lamina total	starch + sugar	E.I.S.*	starch	E.S.*	sugar
	56	40	40	29	16	11
	59	48	36	29	23	19
	48	40	33	26	15	14
Means	54	43	36	28	18	15

% lamina total	% E.I.S.	% E.S.
80	78	83

* E.I.S. Ethanol-insoluble fraction of lamina.
E.S. Ethanol-soluble fraction of lamina.

TABLE II

Changes in amount and radioactivity of starch and sugar in laminae of attached Tobacco leaves kept in air and continuous light after assimilation of $^{14}CO_2$

(Plants depleted of starch. 175 sq. cm. of a single leaf supplied with 111·5 mg. $^{14}CO_2$, activity 167×10^3 c.p.m./mg. C. After complete assimilation of the CO_2, plants placed in air and continuous light of different intensities for 72 hours. See text, p. 336)

		Starch			Sugar (Hexose + Sucrose)		
Light:		Low	Medium	High	Low	Medium	High
		mg./10 leaf disks. duplicates					
Hours after assimilating $^{14}CO_2$	0	1·53	1·84	1·41	1·00	0·81	1·08
		1·28	1·82	1·42	0·74	0·78	0·80
	72	0·68	2·00	5·30	0·51	0·83	0·43
		1·06	2·34	5·33	0·43	0·94	0·30
% loss or gain		−55	+19	+275	−46	+10	−63
		−17					
		Total counts per min. $\times 10^{-3}$/10 *leaf disks. duplicates*					
Hours after assimilating $^{14}CO_2$	0	65	104	66	42	39	45
		60	101	62	32	38	33
	72	18	43	74	2	< 2	< 1
		35	48	73	2	< 2	< 1
% loss or gain		−72	−55	+16	−95	−97	−97
		−42					

sunlight they were not constant. Except for a measured range of low intensities (Table III) the light levels are recorded as high, medium, and low and imply that the total amount of light received can be classified in this way.

One experiment is recorded in full in Table II: this shows effects of exposure for 72 hours to continuous light at three light levels on the amounts and radioactivity of starch and sugar. The plants exposed to each of the different light intensities were sown on different dates, and the fully expanded leaves

used ranged in emergence number from the 16th to the 22nd. In spite of this variability in the history of the leaves used, the amounts of starch and sugar synthesized from the fixed amount of $^{14}CO_2$ were remarkably uniform.

Inspection of Table II shows that the changes in radioactivity of starch depended on the rate of new synthesis as controlled by light. At low light, losses of starch in the two replicates were 55 per cent. and 17 per cent. and these were associated with losses of radioactivity of 72 per cent. and 42 per cent. respectively. At medium light, starch increased by 19 per cent. and activity fell by 55 per cent., and at high light starch increased threefold and loss of radioactivity was prevented, there being instead a small increase. At

TABLE III

Changes in amount and radioactivity of starch and sugar in laminae of attached tobacco leaves in continuous light and in air after assimilation of $^{14}CO_2$. Procedure as in Table II. Low light intensities

		Starch					Sugar			
Light f.c.:		0 dark	250	500	750	1000	250	500	750	1000
		mg./10 leaf disks. Means of duplicates								
Hours after assimilation of $^{14}CO_2$	0	1·42	1·37	1·27	1·40	1·43	0·85	0·80	0·91	0·61
	72	0·15	0·61	1·20	1·75	1·98	0·23	0·40	0·57	0·62
Mean % loss or gain		−89	−56	−5	+25	+38	−73	−50	−37	+2
		Total counts per min. $\times 10^{-3}$/10 *leaf disks. Means of duplicates*								
Hours after assimilation of $^{14}CO_2$	0	49	67	59	69	72	37	36	48	33
	72	1	17	23	50	45	< 1	< 1	1	1
Mean % loss		98	74	61	28	38	98	97	98	96

all three light intensities activity virtually disappeared from sugar. It is concluded that starch dissolution proceeds in light, and also that there is continuous accretion and depletion of sugar resulting, in time, in complete replacement. At low and medium light intensities the partial exchange of starch is detectable by the differences between the percentage changes in amounts and activities, and it is noteworthy that when the amount of starch remained fairly constant about half the original material was replaced by new synthesis. The retention of activity with high light suggests that exchange may be largely confined to the outer layers of starch grains. If inactive layers were rapidly superimposed on the radioactive starch in high light then exchange would soon be between inactive molecules only and so not observable. The replacement of sucrose and hexose is presumably accounted for mainly by removal in translocation, and possibly some by respiration in light with replenishment by new assimilate. Only at high light is there any evidence that the activity of starch increased at the expense of sugar, after placing the plants in air (Tables II and IV).

Results of two further experiments are presented in Tables III and IV.

On the one hand (Table III) a range of light intensities below 1,000 f.c. was used and on the other (Table IV) the high light intensity was provided by sunlight. With the low intensities (Table III) losses of starch formed a sequence from an 89 per cent. loss in darkness to a gain of 38 per cent. at 1,000 f.c., accompanied by losses of 98 per cent. and 38 per cent. respectively in activity. As overall starch loss diminished with rising light intensity, the activity lost also diminished but not proportionately. At about 500 f.c. the starch content remained constant, while half the activity was lost (cf. Table

TABLE IV

Changes in amount and radioactivity of starch and sugar in laminae of attached tobacco leaves in continuous light and in air after assimilation of $^{14}CO_2$. *Procedure as in Table II. High light intensities*

(Light 1,000 to 2,000 f.c. plus sunlight)

		Starch			Sugar		
		mg./10 leaf disks.			*Means of duplicates*		
Hours after assimilating $^{14}CO_2$	0	1·64	1·69	1·69	1·02	0·81	1·16
	72	4·10	8·48	10·45	0·82	0·94	0·88
Mean % gain or loss 7–11 days		+150 4·28	+402 6·08	+518 6·12	−20	16	−24
		Total counts per min. $\times 10^{-3}$/10 *leaf disks. Means of duplicates*					
Hours after assimilating $^{14}CO_2$	0	69	76	72	44	33	51
	72	69	79	90	1	1	1
Mean % gain or loss 7–11 days		none 42	+4 63	+25 67	−97	−97	−99

II). In sunlight (Table IV) up to fivefold increases in starch were observed, accompanied by increases of activity up to 25 per cent. The results of these two experiments confirm those of the experiment recorded in Table II.

After the period of 72 hours in continuous light, plants from the high light treatments (Table IV), were kept from 7 to 11 days in the greenhouse under prevailing daylight conditions, and were not illuminated at night. At the end of these times the onset of yellowing was approaching. The further changes in the amount and activity of the starch are appended to Table IV. In all three leaves starch content was still high and amounted to at least double that of the original radioactive starch, in spite of losses at night. Nevertheless there were losses of activity of 20 per cent. to 40 per cent. It seems likely therefore that if the leaves remained green all the radioactive material would eventually be slowly replaced. This slow removal of activity might be explained if there were a slow exchange of material throughout the grains in addition to the fast exchange from outer layers. Another possibility would be complete loss of the inactive portion at night from some of the small grains, resulting in exposure of a radioactive layer.

Finally, $^{14}CO_2$ was supplied to leaves already containing starch, thus forming grains with a radioactive outside layer. The plants were then kept in continuous light at three different intensities but for 24 hours only instead of 72 hours as before. The results were very similar to those set out in Table II and so are not presented here in detail. At low light the overall loss of starch exceeded in amount that of the radioactive layer added, and 84 per cent. of the activity was lost (cf. Porter *et al.*, loc. cit.). At medium light, without change in amount, 55 per cent. of activity was lost and at high light the amount increased by 86 per cent. without change in activity. Again, nearly all the activity disappeared from sugars. Thus when radioactive starch was added as an outside layer to inactive material, activity loss in light in 24 hours was comparable in amount to that lost in 72 hours, when the same amount of active material constituted the whole grain (Table II). Such a difference in the rate of change could be interpreted by supposing that when a given amount of active material was spread over a larger area by forming only an outside layer instead of the whole grain a higher proportion was readily accessible for exchange.

Speculation on the mechanism of exchange would be premature. The purpose here is merely to provide evidence for the view that in light there is continuous dissolution of starch and that continuous depletion of leaf sugars, sucrose, and hexose takes place by translocation or respiration. Simultaneous replacement by new photosynthate maintains, or even increases the level of starch and sugar in the tissue.

We are indebted to Professor H. K. Porter, F.R.S., for suggesting the problem and for continued interest and encouragement.

LITERATURE CITED

Jones, H., Martin, R. V., and Porter, H. K. (1959). Translocation of ^{14}Carbon in tobacco following assimilation of ^{14}Carbon dioxide by a single leaf. *Ann. Bot.*, N.S. **23**, 493.

Porter, H. K., Martin, R. V., and Bird, I. F. (1959). Synthesis and dissolution of starch labelled with ^{14}Carbon in tobacco leaf tissue. *J. Exp. Bot.* **10**, 264.

Growth Substances in the Roots of *Vicia faba*[1]

A. N. LAHIRI[2] AND L. J. AUDUS

Bedford College, London University

Received 13 May 1960

SUMMARY

An investigation has been made into the growth regulators present in ethanol extracts of the seedling roots of *Vicia faba* after separation on paper partition chromatograms, using segments of *Avena* coleoptiles and mesocotyls and of *Pisum sativum* roots as biological assay material.

Acetonitrile purification shows the presence of at least three auxins running in isobutanol: methanol: water, at Rfs of 0–0·25, 0·4–0·6, and 0·65–0·95; the latter may represent two different auxins. A similar, but clearer, picture is shown by the ether-soluble acid fraction. Here an auxin at Rf 0–0·25 also stimulates root growth and could be 'accelerator α'. A second at Rf 0·35–0·65 is an indole compound which inhibits root growth and does not seem to be IAA. A third at Rf 0·8–1·0 is also a root-growth inhibitor and gives no indole reaction. The 'inhibitor β' complex was demonstrated (Rf 0·65–0·85) together with a number of other inhibitors at lower Rf value.

The ether-soluble neutral component also contains auxins or auxin precursors.

The water-soluble, ether-insoluble fraction contains four readily interconvertible substances with auxin properties. They all appear to inhibit root growth and give no indole reaction.

INTRODUCTION

A PREVIOUS report (Audus and Gunning, 1958) has shown that in the roots of *Pisum sativum* there exists a complex of growth substances including, in addition to the commonly found ether-soluble acid auxins giving indole colour reactions, some easily interconvertible water-soluble, ether-insoluble auxins, apparently not indole compounds. In preliminary experiments designed to investigate the effects of certain manipulations on the distribution of such hormones in roots, it was found that *Vicia faba* was more satisfactory for the purpose than *Pisum sativum*. Hence similar studies have been made of growth substances in this species, the results of which are set out in this paper.

MATERIAL AND GENERAL METHODS

Extracts were made from seedling roots of *Vicia faba* var. 'Green Leviathan'. These were grown first in moist sterile sand for 3 days in the dark at 25° C. As large as possible a sample was then selected for uniform root length,

[1] The experimental work described in this paper comprised part of a thesis presented by A. N. Lahiri for the Ph.D. degree of the University of London.

[2] Present address: Radio-Chemical Laboratory, Bose Institute, 93/1, Upper Circular Road, Calcutta 9, India.

transferred to aerated water-culture tanks of earthenware, and further grown under artificial light (500 ft. candles) in a 14-hour day at 25° C. The roots were darkened during this growth period. The methods of extraction, chromatography, and bioassay of auxins were all identical with those used in the previous work (Audus and Gunning, 1958). When the presence of substances active on root elongation was being studied, the pea-root segment assay of Audus and Thresh (1953) was used.

Chromatogram strips were always cut into twenty transverse segments for assay, the fiducial limits of which were calculated from the means of ten control samples grown on similar strips of solvent-washed paper.

RESULTS

1. *Acetonitrile soluble substances*

As described for the pea-root work, cold ethanol extracts were purified by the acetonitrile method of Nitsch (1955, 1956). Fig. 1 illustrates the results obtained. Irrespective of the test used, there seem to be four clear growth-promoting regions on these chromatograms. Firstly, at Rf = 0–0·25, there is a zone particularly effective on the mesocotyl, and this may correspond to the same substance as AP (*i*) found in pea roots. This substance is also probably identical with the 'accelerator α' of Kefford (1955). This is further supported by the fact that the substance in pea-root extracts consistently increases the extension growth of pea-root segments, as shown in the composite diagram of Fig. 2 where the pooled results of five separate analyses of the acetonitrile fractions of extracts are shown.

The second auxin zone falls between Rf 0·4 and 0·6, at or slightly below the position of the IAA marker spot. This corresponds with the substance AP (*ii*) of pea roots and has been suspected of being IAA itself (see later).

In very strong contrast to the results from peas, marked stimulation is observed in *Vicia* root chromatograms at Rf values above 0·6–0·7, where responses tended to exceed those due to AP (*ii*) and show two promoting spots. In this region pea-root chromatograms gave results which indicated the presence of both auxins and inhibitors. Undoubtedly, the *Vicia* auxins in this region are either more active or present in higher concentrations than the corresponding pea auxin (AP (*ii*)) but there is little net inhibition in this region, even with the coleoptile test, which is sensitive to inhibitors (Fig. 1C and D). As will be seen later, the ether-soluble fraction of ethanol extracts shows only one promoting spot in this region, and it is therefore suspected that we may be dealing here in the acetonitrile fraction with a very broad spot of such a single auxin spreading from Rf 0·65 to Rf 0·95 and that this has been 'split' by an inhibitor running at about Rf 0·8 in this solvent. This inhibitor could be the same as that present in pea extracts (AI (*ii*)) and might consist of one or more components of the 'inhibitor β' complex (Housley and Taylor, 1958). In these few tests no indication was obtained of inhibitions at lower Rf values (cf. AI (*i*) of pea).

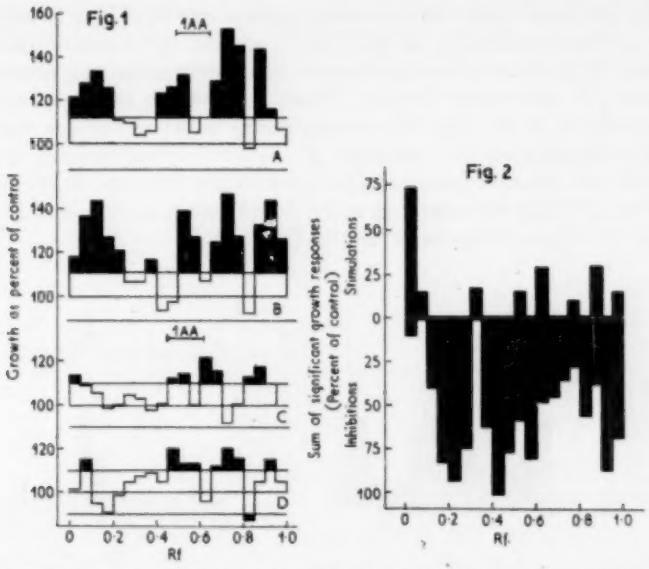


FIG. 1. Chromatograms (isobutanol: methanol: water solvent) of the acetonitrile-soluble fraction of ethanol extracts of *Vicia faba* roots. A and B *c.* 50 g. from 14-day-old seedlings assayed by *Avena* mesocotyl segment test. C and D *c.* 35 g. from 10-day-old seedlings assayed by the *Avena* coleoptile segment test. The three horizontal lines represent control mean and 5% fiducial limits of assay respectively.

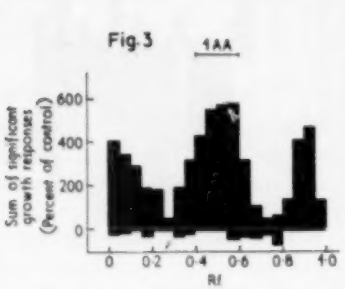
FIG. 2. Composite diagram from five separate chromatograms (isobutanol: methanol: water solvent) showing Rf values of acetonitrile-soluble substances in ethanol extracts of *Pisum sativum* roots as assayed by the pea-root segment test. All *significant* growth responses (per cent. increase over control) have been summed.

2. *Ether-soluble substances*

Cold ethanol extracts were evaporated at 35° C. in a flash evaporator under vacuum until all the ethanol had come off. The aqueous residue was then acidified to pH 3 with N/10 sulphuric acid. This was then shaken with four successive aliquots of ether. The pooled ether fractions were then partitioned against 5 per cent. $NaHCO_3$ to remove acid auxins. These were then removed from the bicarbonate solution by reacidifying to pH 3 and repartitioning against ether. This acid-auxin extract was then reduced to dryness *in vacuo*, the residue taken up in a small quantity of ether and spotted on a chromatogram. The neutral-ether fraction was similarly treated.

(*a*) *Acid auxins.* Chromatograms have been assayed predominantly by the mesocotyl test. They show a remarkable uniformity of character, and this has been taken as justification for combining the results in Fig. 3, where the sums of all *significant* responses for 21 such chromatograms are plotted against the relevant Rf intervals.

There are three clear-cut promoting regions corresponding closely with the AP (*i*) ('accelerator α'), AP (*ii*) ('IAA'), and AP (*iii*) zones described for pea roots. There is no indication whatever of a fourth promoting zone in high Rf values (cf. acetonitrile fraction). There is, however, consistent evidence of an inhibitor at Rf 0·55–0·8, corresponding to AI (*ii*) of pea roots and probably representing the 'inhibitor β' complex. When assayed with the coleoptile test, which is more sensitive to inhibitors, this zone shows up more positively, although the responses to the AP (*ii*) zones are much smaller and there is virtually no response to AP (*iii*) (Fig. 4A).



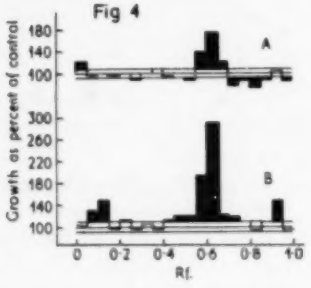
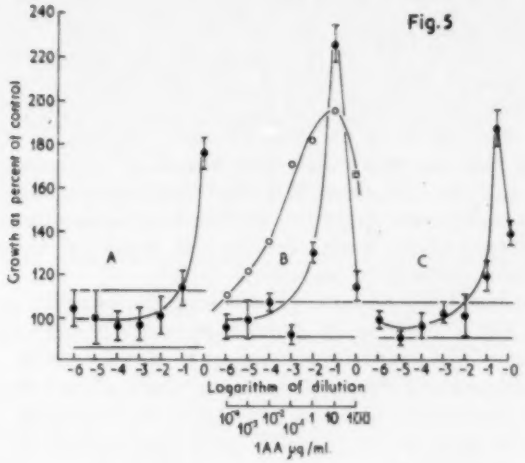


FIG. 3. Composite diagram from twenty-one separate chromatograms (isobutanol: methanol: water solvent) showing Rf values of acid, ether-soluble substances in ethanol extract of *Vicia faba* roots as assayed by the *Avena* mesocotyl segment test. All *significant* growth responses (per cent. increase over controls) have been summed.

FIG. 4. Comparison of *Avena* coleoptile segment assay (A) and *Avena* mesocotyl segment assay (B) of replicate chromatograms of ether-soluble acid substances in ethanol extracts of 40 g. of roots of 11-day-old seedlings of *Vicia faba* (Solvent: isobutanol: methanol: water). The three horizontal lines represent means and 5% fiducial limits of assay respectively.

In an attempt to throw further light on the nature of these substances, dilution-response curves have been plotted for them in both mesocotyl segment and root segment tests. For this purpose two strictly comparable extracts, one from 40 g. and the other from 150 g. of roots, were chromatographed concurrently on the same paper. The strip corresponding to the lesser quantity of extract was assayed by the mesocotyl test to indicate the active regions of the other strip. These active regions of the second chromatogram were then cut out and eluted by shaking in the dark for 5 hours in 10 ml. ethanol. The eluates were then taken down to dryness under reduced pressure and the residues each redissolved in 4 ml. of bioassay medium. One in 10 serial dilutions of each were then made in the same medium and the activities measured in the two tests. These results of the mesocotyl assay are shown in Fig. 5.

With all three of these auxins, activity changes much more rapidly with dilution than is the case with synthetic auxins such as IAA, for which substance a concentration-activity curve has been superposed on that for AP (*ii*)



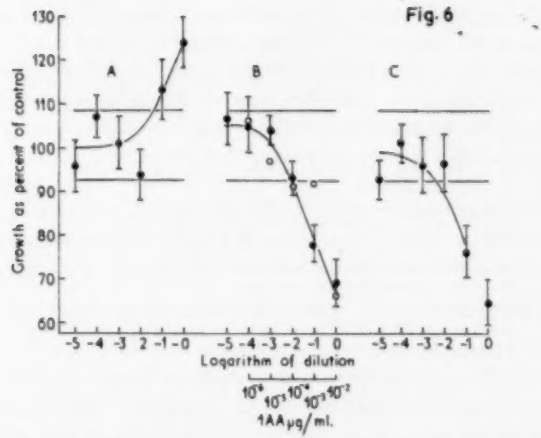


FIG. 5. Dilution–growth response curves for the three ether-soluble acid auxins from roots of *Vicia faba*, in the *Avena* mesocotyl segment assay. A. AP (*i*) at Rf 0–0·2. B. AP (*ii*) at Rf 0·35–0·6. C. AP (*iii*) at Rf 0·8–1·0. Each black point represents the mean response of a large sample of 30–40 segments, the vertical lines reaching to ± standard deviation on either side. The parallel lines show the variability of control segments (mean ± standard deviation). For AP (*ii*) the response curve of IAA has been superposed (open circles) so that the optimal concentrations of the two substances coincide.

FIG. 6. Dilution–growth response curves for the three ether-soluble acid auxins from roots of *Vicia faba* in the pea-root segment test. All details as for Fig. 5.

in the figure. This holds both for sub- and supra-optimal concentrations, so that auxin activity is confined to a much narrower range of concentrations than for the synthetic IAA. In the case of AP (*ii*), the increased extension induced by optimal concentrations is very significantly greater than that normally induced by optimal (10 p.p.m.) IAA concentrations, which suggests that AP (*ii*), although it has the same Rf value, may not be IAA. But it is dangerous to read too much into mere shapes of concentration-response curves, since we have no evidence that other components of the extract eluted from the chromatograms might not be distorting these curves by unduly depressing activity in the lower concentration ranges, or by acting synergistically at the optimum concentration.

Similar concentration response curves using the pea-root segment assay are shown in Fig. 6. These are of eluates from similar chromatograms involving rather larger quantities of roots (220 g. as compared with 150 g. in the above mesocotyl assays). AP (*i*) causes a stimulation of root extension which is about half that produced by corresponding concentrations in mesocotyl segments. It has thus the properties of 'accelerator α' (Kefford, 1955) and is probably identical with that substance. AP (*ii*) strongly inhibits root extension. The decrease of inhibition on dilution follows a curve very similar in shape to that of the IAA curve (superposed in Fig. 6) but the striking feature of this relationship is that the inhibition is very much smaller than would have been expected from the responses of the mesocotyl and the known relative sensitivities of mesocotyl and root to IAA. Thus to give the same root-growth inhibition as a 1 to 10 dilution of this quantity of AP (*ii*), a concentration of IAA of approximately 2×10^{-3} mg./L would be required. A similar dilution of the same quantity of AP (*ii*) gives maximal stimulation of mesocotyl growth for which an IAA concentration of 10 mg./L is necessary. Thus, judged on a basis of equal effectiveness of both substances on mesocotyl growth, IAA would seem to be $\frac{10}{2 \times 10^{-3}}$ or 5,000 times more active than AP (*ii*) on root growth. This is much stronger evidence that AP (*ii*) is not IAA.

AP (*iii*), like AP (*ii*), inhibits root growth to about the same extent and shows a similar dilution-response curve. However, since the region eluted probably contained some of the 'inhibitor β' complex as well as AP (*iii*), this complication vitiates further theorizing. Separation of these components must come before we can further characterize them. This may not be difficult, since preliminary experiments with two-way paper chromatography show that AP (*i*), AP (*ii*), and AP (*iii*) all run between Rf 0·5 and 0·9 in 70 per cent. ethanol, whereas 'inhibitor β' runs at Rf 0·1–0·35 (Fig. 7). This method seems to have shown up clearly another auxin (*a*) running little in either solvent and three additional inhibitors (*b*, *c*, and *d*) (Fig. 7) whose presence was also indicated occasionally on one-way chromatograms (see Fig. 3). Unfortunately the intolerable amount of labour involved in assaying these chromatograms offsets the greatly improved separation obtained with them and precludes their use in routine quantitative growth substance estimations.

One-way chromatograms of extracts of about 150 g. of roots have been sprayed with Ehrlich's reagent, when pinkish-violet spots have appeared at Rfs 0·2–0·3 and 0·45–0·6. The second of these corresponds closely with the peak of AP (*ii*) and indicates strongly that it is an indole compound. The first falls between AP (*i*) and AP (*ii*), where there is little or no growth activity, and may indicate the presence of an inactive indole compound.

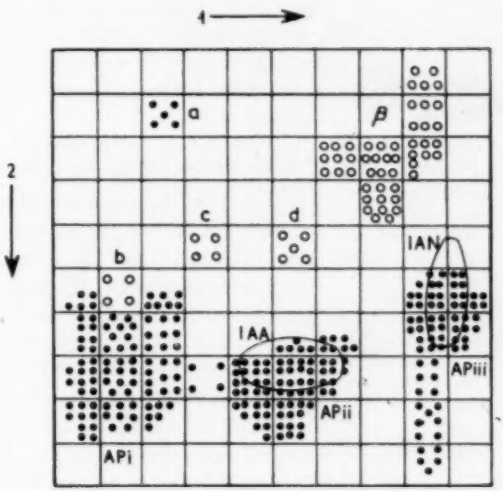


FIG. 7. Two-way chromatogram of the ether-soluble acid growth substances in ethanol extracts of *Vicia faba* roots, assayed by the *Avena* coleoptile segment test, each of the 100 squares being separately assayed. Where *significant*, (above 5% fiducial limits), the responses have been marked in the relevant square, the total number of points per square representing the degree of response ((per cent. change from control)/4).

Black points = stimulation. Open circles = inhibition. The running positions of IAA and IAN on similar chromatograms are ringed. The running solvents used were

1. direction, isobutanol: methanol: water: : 80: 5: 15;
2. direction, ethanol: water: : 7: 3.

(*b*) *Neutral auxins.* Neutral ether-soluble auxins were also assayed by the mesocotyl technique after separation on a paper chromatogram. Very inconsistent results were obtained by the use of the isobutanol: methanol: water solvent, due apparently to gummy materials interfering with the subsequent assay. By using water as a running solvent these gummy materials stayed at the starting line and gave much cleaner chromatograms, but with much lower activity. The picture obtained from such studies is nothing like as clear cut as with the acid auxins. The pooled results of a number of such

chromatograms are shown in Fig. 8. In the zone Rf 0–0·2 an accelerator appears consistently. This seems to be an indole compound since a deep pinkish-violet colour consistently develops on spraying this region with Ehrlich's reagent. It runs at approximately the same Rf as indole-3-acetonitrile (Rf 0·4; Larsen, 1955) but is probably not that substance, which gives a characteristic greenish-yellow colour with Ehrlich's reagent. A similar accelerator, rather more widely spread, appears between Rf 0·7 and 1·0 and there is the suggestion of a third between Rf 0·4 and 0·6. Apart from these qualitative observations no further work has yet been done on this fraction.

Fig. 8

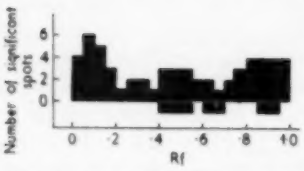


FIG. 8. Composite diagram from six separate chromatograms (isobutanol: methanol: water solvent) showing Rf values of ether-soluble neutral auxins in ethanol extracts of *Vicia faba* roots, as assayed by *Avena* mesocotyl segment test. Unlike Figs. 2 and 3, each black square represents simply the presence of significant activity at the relevant Rf value.

3. *Water-soluble ether-insoluble substances*

These were the substances remaining in the aqueous residue of the alcohol extract after repeated extractions with ether at pH 3. This was just neutralized with barium hydroxide, filtered, and then concentrated under vacuum at 35° C. in a flash evaporator. In preliminary work we studied the efficiency of various solvents in extracting active substances from the resultant syrup. Petrol ether extracted little active material. Benzene extracted small quantities of inhibitors running at high Rf values in the isobutanol: methanol: water solvent. Acetonitrile extracted both accelerators and inhibitors in small amounts. Only ethanol and chloroform produced consistent chromatographic patterns of four growth accelerators extracted in relatively large quantities. Fig. 9 shows two such chromatograms of material extracted by these two solvents. It will be seen that, as for the corresponding fractions of pea-root extracts, there are four significant promotion spots using the *Avena* mesocotyl test. In ascending Rf values in this solvent they occur in approximately the same positions as the WP (*i*), WP (*iv*), WP (*ii*), and WP (*iii*) respectively of pea roots. In addition to the *Avena* mesocotyl assay, the effects of these substances on *Avena* and *Triticum* coleoptile and pea-root segments were also determined and the results of such tests using extracts from the same quantities (*c.* 10 g.) of similar root samples are shown in Fig. 10. All three organs are sensitive to the same four compounds. In roots they all inhibit growth; in coleoptiles they all promote it and it is somewhat surprising that, unlike the situation with IAA, coleoptiles seem on the whole *more* sensitive to these compounds than the mesocotyl.

The same peculiar interconversion behaviour is shown by these bean-root substances as by the corresponding substances in peas. This was demonstrated after running on the same paper two chromatograms of extracts from 5·6 and 11·3 g. respectively of roots from the same sample. The paper was then split longitudinally and the chromatogram for the smaller quantity of

Fig.9

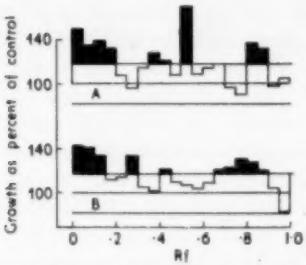


FIG. 9. Chromatograms (isobutanol: methanol: water solvent) of water-soluble, ether-insoluble auxins in ethanol extracts of 11 g. of 15-day-old *Vicia faba* roots assayed by the *Avena* mesocotyl segment test. A. Ethanol extract of residual syrup. B. Chloroform extract of residual syrup. The three horizontal lines represent control means and 5% fiducial limits of assay respectively.

Fig.10

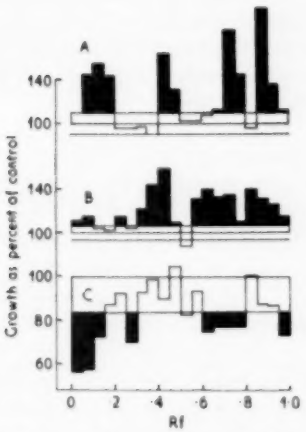


Fig.11

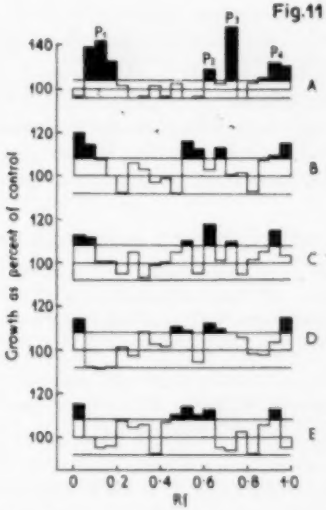


FIG. 10. Chromatograms (isobutanol: methanol: water solvent) of the water-soluble, ether-insoluble auxins in ethanol extracts of 10 g. of *Vicia faba* roots assayed by *Avena* coleoptile (A), *Triticum* coleoptile (B), and pea-root (C) segment test. The parallel lines indicate control means and 5% fiducial limits of assay respectively.

FIG. 11. Chromatograms (isobutanol: methanol: water solvent) showing the interconvertibility of the water-soluble, ether-insoluble auxins in ethanol extracts of *Vicia faba* roots. All assays by *Avena* mesocotyl test. The parallel lines represent the control means and 5% fiducial limits of assay respectively.

A. Primary chromatogram of extract from 25 g. roots.

B, C, D, and E. Chromatograms from zones P_1, P_2, P_3, and P_4 respectively of the primary chromatogram.

extract assayed by the mesocotyl test. This gave a pattern as shown in Fig. 11A. The zones of the second chromatogram corresponding to the four promoter spots were then cut out and eluted, as previously described for the pea-root experiments, concentrated *in vacuo* at 35° C., and then separately chromatographed. The results of the subsequent assays are shown in Fig. 11B–E respectively. In each case separate promoter spots appeared as in the first chromatogram. Undoubtedly there exists here as in pea roots a system of easily and spontaneously interconvertible substances with marked auxin activity. They have not yet been shown to give any of the usual indole colour reactions. Similar elutions followed by chromatography have been carried out for the three acid ether-soluble auxins from bean roots (AP (*i*), AP (*ii*), and AP (*iii*)) but there was no sign of a similar interconversion since for each substance only a single spot occurred on the paper at the proper Rf value.

CONCLUSIONS

From these results it would seem that roots of *Vicia faba* possess the same spectrum of auxins as roots of *Pisum sativum*. What differences there are seem to be of degree rather than kind and this is not surprising in view of their taxonomic affinities. However, doubts will remain until exact chemical characterization has been made. Even so, enough has been demonstrated to indicate that IAA, even if it is present, may play only a subsidiary role in the growth control of roots, where many other substances with similar physiological properties are present in considerable quantities.

LITERATURE CITED

AUDUS, L. J., and GUNNING, B. E. S. (1958). Growth substances in the roots of *Pisum sativum*. *Physiol. Plantarum* **11**, 685–97.

—— and THRESH, R. (1953). A method of plant growth substance assay for use in paper partition chromatography. Ibid. **6**, 451–65.

HOUSLEY, S., and TAYLOR, W. C. (1958). Studies on plant-growth hormones. VI. The nature of inhibitor-β in potato. *J. Exp. Bot.* **9**, 458–71.

KEFFORD, N. P. (1955). The growth substances separated from plant extracts by chromatography I. Ibid. **6**, 245–55.

LARSEN, P. (1955). Growth substances in higher plants. *Modern Methods of Plant Analysis* (Paech, K., and Tracey, M. V., ed.), Berlin: Springer Verlag, Vol. III. pp. 565–625.

NITSCH, J. P. (1955). Free auxins and free tryptophan in the strawberry. *Plant Physiol.* **30**, 33–39.

—— (1956). Methods for the investigation of natural auxins and growth inhibitors. *The Chemistry and Mode of Action of Plant Growth Substances*. Ed. Wain, R. L., and Wightman, F., Butterworths, London. pp. 3–31.

Growth Response of *Triticum* Coleoptiles to Non-dialysable Milk Fractions[1]

J. E. GANDER[2]

Department of Chemistry, Montana State College, Montana, U.S.A.

Received 27 November 1959

SUMMARY

Dialysed skimmed cows' milk was shown to promote the elongation of *Triticum vulgare* var. 'Yogo' coleoptiles. The optimum concentration of skimmed milk was found to be one part to 200 parts distilled water. Milk dialysate was shown to inhibit elongation but after the cations were removed to have no influence upon elongation.

Gelatin, β-lactalbumin, phosphovitin, and glycoprotein fraction IV each present at a concentration of 30 mg./100 ml., increased coleoptile elongation about 20 per cent. when compared to a distilled-water control. Proteose-peptone and α-lactalbumin caused a slight inhibition in elongation.

These results are discussed in terms of possible factors such as bacterial contamination or adsorbed growth-promoting substances as agents causing these effects.

INTRODUCTION

PRELIMINARY investigations by Iverson (1947) and Iverson and Johnson (1948) indicated that the application of skimmed cows' milk to soil in which tomatoes were growing caused the plants to be taller, have a more fibrous root system, greater leaf area, and increased early and total yields of fruit. In these and other preliminary studies it was not possible to determine whether (*a*) skimmed milk contains a plant-growth regulator, (*b*) the responses observed were due to a more favorable nutritional environment caused by the milk or a combination of the milk and soil microflora, or (*c*) a plant growth regulatory substance produced by the soil microflora caused the observed responses. Their data gave no indication whether skimmed milk contains factors which would give a positive response in a standard plant growth-regulator bioassay.

Investigations conducted by Housley and Bentley (1954, 1956, 1956) and Kefford (1953, 1955) suggested there may be more than one ether-soluble substance present in plant tissue which will promote an increased rate of elongation in etiolated *Avena* or *Triticum* coleoptiles. It has been suggested that these materials may function in the regulation of plant growth; however, Booth (1958) has recently shown that sucrose or tryptophane, materials not

[1] Montana Agricultural Experiment Station, Journal Series Number 459.

[2] Present address: Department of Agricultural Biochemistry, The Ohio State University, Columbus 10, Ohio.

normally considered as plant-growth regulators, will also promote an increase in the rate of elongation of *Avena* coleoptiles. The influence of proteins upon plant-growth regulator bioassays is unknown.

This paper reports the results of experiments undertaken to determine whether various fractions of milk have any influence upon a standard plant growth assay.

MATERIALS AND METHODS

Materials. Egg albumin, gelatin, α-lactalbumin, bovine serum albumin, ovalbumin, phosphovitin, β-lactalbumin, glycoprotein (fraction IV), and proteose-peptone were obtained from Nutritional Biochemicals Corporation. Skimmed milk was obtained from the Department of Dairy Industry, Montana State College.

Plant-growth regulator bioassay. Triticum vulgare variety 'Yogo' was grown at 27° C. in sand and irradiated with red light for approximately 72 hours. Etiolated plants 18 to 22 mm. long were used as the assay organism (Bentley and Housley, 1954). Ten-mm. sections were cut 3 mm. from the tip, randomized, and 20 sections placed in each petri-dish containing 20 ml. of liquid. Each treatment was run in triplicate and the average of the three treatments reported. The petri-dishes were placed under a red light for 18 hours at 27° C., followed by measuring the sections to the nearest 0·2 mm. Distilled water was used as one control and distilled water containing 1 mg./l. 2,4-D was used as the other control, giving responses of $3{\cdot}0 \pm 0{\cdot}2$ mm. and $8{\cdot}0 \pm 1{\cdot}1$ mm. respectively. All results are expressed as per cent. elongation compared to the distilled-water control.

Milk-fraction additions were made to the distilled water in the quantities indicated.

Preparation of milk fractions. Dialysed skimmed milk was prepared by dialysing skimmed cows' milk against continuously running tap water at 9° C. for 48 hours. Skimmed-milk dialysate was prepared by dialysing 100 ml. of distilled water against 5 litres of skimmed milk for 48 hours at 2°–4° C., and the contents within the dialysis tubing used as the skimmed-milk dialysate. Anion-low milk dialysate was prepared by passing 10 bed volumes of skimmed-milk dialysate through a Dowex-1-OH resin, while the cation-low milk dialysate was prepared by passing a similar volume of skimmed-milk dialysate through a Dowex-50-H resin and using the eluates after adjusting to pH 6·0 with NaOH or HCl. Aromatic compounds found in the milk dialysate were isolated by treating 100 ml. of skimmed-milk dialysate with 5 g. of Darco G, followed by removal of the charcoal by centrifugation. The charcoal was washed three times with distilled water, transferred to a chromatographic column, and eluted with 7 per cent. phenol as described by Asatoon and Dalgliesh (1956). The elute was lyophylized to dryness, water added, and relyophylized until no phenol could be detected. The residue was dissolved in 100 ml. of distilled water. All skimmed-milk dialysates were stored at −18° C. until used.

EXPERIMENTAL RESULTS

The data in Table I show the influence of various milk fractions upon the elongation of *Triticum* coleoptiles. Dialysed skimmed milk increased the elongation of *Triticum* coleoptiles while the milk dialysate inhibited elongation. The passage of the milk dialysate through a cation-exchange resin

TABLE I

Effect of Milk Fractions upon Elongation of Etiolated Wheat Coleoptiles

Fraction[1]	Elongation compared to control, %
Skimmed milk	90±18
Dialysed skimmed milk	137±16
Skimmed-milk dialysate	64±23
Anion-low milk dialysate	67±21
Cation-low milk dialysate	97±19
Aromatic materials in milk dialysate	93±17
Lactose (0·01 M.)	112±15

[1] Aliquots equivalent to materials in 0·10 ml. of skim milk were added to 20 ml. of distilled water.

removed the inhibitory material or materials. Calcium ion, a major constituent of a milk dialysate, has been shown by Tagawa and Bonner (1957) to increase cell wall elasticity and plasticity; the calcium ion in the dialysate would be expected to inhibit elongation. The aromatic materials removed from the activated charcoal apparently had little effect upon coleoptile elongation as shown in this table. Ordin and Bonner (1957) have presented evidence that galactose inhibits elongation and respiration of *Avena* coleoptile sections; however, 0·01 M. lactose had no inhibitory effect upon *Triticum* coleoptile elongation.

The optimum concentration of dialysed skimmed milk in promoting coleoptile elongation was sought. Table II shows the effect of various concentrations of dialysed skimmed milk upon coleoptile elongation. The addition of 0·10 ml. of dialysed skimmed milk gave the maximum growth response.

TABLE II

Effect of Concentration of Dialysed Skimmed Milk upon Elongation of Etiolated Wheat Coleoptiles

Ml. of dialysed skimmed milk added to 20 ml. H_2O	Elongation compared to control, %
0·02	112±18
0·04	127±14
0·10	137±16
0·20	123±19

It appeared that the non-dialysable fraction of skimmed milk was responsible for the observed growth response, therefore other proteins were assayed to determine whether a growth response resulted. In Table III the various

TABLE III

Effect of Proteins upon Elongation of Etiolated Wheat Coleoptiles

Materials assayed[1]	Elongation compared to control, %
Gelatin	128±13
Glycoprotein (Fraction IV)	124±16
Phosphovitin	120±13
β-lactalbumin	118±15
Bovine serum albumin	109±11
Egg albumin	106±20
Ovalbumin	103±17
Casein	96±7
α-lactalbumin	94±8
Proteose-peptone	87±12

[1] All materials were added to give a final concentration of 0·03%.

proteins assayed are listed. All proteins were assayed at a concentration of 6 mg. per 20 ml. of solution. In dishes containing either gelatin, glycoprotein (fraction IV), phosphovitin or β-lactalbumin, the coleoptile elongation was greater than either the control or the other proteins assayed.

DISCUSSION

The data presented indicate that some proteinaceous or protein bound material increased the elongation of wheat coleoptiles as much as 30 per cent. This effect appears to be a rather non-specific one, with a phosphoprotein, glycoprotein, and protein high in glycine and proline giving the largest response.

Benton *et al.* (1957) reported the presence of a growth factor for chicks in gelatin. Whether this is related to the growth response observed in *Triticum* coleoptiles when assayed in the presence of gelatin is not known. It is known that some organic compounds bind to proteins even after being recrystallized several times, as witnessed by Donaldson and Nason (1957) with bovine serum albumin. Our data give no evidence as to whether materials which will increase coleoptile elongation are bound to the protein or whether the response observed is due entirely to the proteinaceous molecules. It is possible that some peptide bound to the surface of the protein caused the elongation responses observed. Woolley (1946) has reported that various peptides influence the growth of *L. casei*; however, growth of an organism and coleoptile elongation are undoubtedly quite different physiological processes. Bacterial contamination could easily account for the observed results, if hydrolytic enzymes were releasing tryptophan from the protein; however, all proteins did not elicit a positive response in *Triticum* coleoptiles. This may be due to the fact that some proteins are more susceptible to proteolytic attack by bacterial enzymes. Another contributing factor could be that the percentage tryptophan in the proteins varies.

With the widespread use of herbicides in arresting growth of certain weeds it is entirely possible that enough of this type of material remains bound to

the protein in milk, even after extensive dialysis, to cause increased elongation. This does not seem too plausible an explanation of the data as the dialysed skimmed milk was prepared twenty-four times over a two-year period and essentially the same results were obtained each time, while one might expect seasonal differences if the responses were due to absorbed herbicides.

Maximum elongation was obtained with 200 mg. of protein per litre in the dialysed skimmed milk. Higher concentrations inhibited growth.

These experiments point out the necessity for caution in interpreting data obtained using standard coleoptile cylinder elongation in terms of plant-growth regulators, especially when assaying heterogeneous biological material.

LITERATURE CITED

ASATOON, A., and DALGLIESH, C. E. (1956). The use of deactivated charcoals for the isolation of aromatic substances. *J. Chem. Soc.* 2291–9.

BENNET-CLARK, T. A., and KEFFORD, N. P. (1953). Chromatography of the growth substances in plant extracts. *Nature*, **171**, 645.

BENTLEY, J. A., and HOUSLEY, G. B. S. (1954). Bioassay of plant growth hormones. *Physiol. Plant.*, **7**, 405–19.

BENTON, D. A., SPIVEY, H. E., and ELVEHJEM, C. A. (1957). Properties of substances in gelatin which stimulate the growth of chicks fed amino acid diets. *Arch. Biochem. Biophys.* **70**, 491–8.

BOOTH, A. (1958). Non-hormonal growth promotion shown by aqueous extracts. *J. Exp. Bot.* **9**, 306–10.

DONALDSON, K. O., and NASON, A. (1957). Interrelationship between vitamin E and lipide cofactor in the cytochrome c reductase system. *Proc. Natl. Acad. Sci. U.S.* **43**, 364–9.

HOUSLEY, G. B. S., and BENTLEY, J. A. (1956). Studies in plant growth hormones IV. *J. Exp. Bot.* **7**, 219–51.

BRITTON, G., HOUSLEY, S., and BENTLEY, J. A. (1956). Studies in plant growth hormones V. Ibid. **7**, 239–51.

IVERSON, V. E. (1947). Effects of skim milk on the growth and yields of tomatoes. *Proc. Am. Soc. Hort. Sci.* **49**, 263.

—— and JOHNSON, L. H. (1948). Effects of milk products on the growth and yields of vegetables. Ibid. **51**, 362–6.

KEFFORD, N. P. (1955). The growth substance separated from plant extracts by chromatography I. *J. Exp. Bot.*, **6**, 245–55.

ORDIN, L., and BONNER, J. (1957). Effect of galactose on growth and metabolism of *Avena* coleoptile sections. *Plant Physiol.* **32**, 212–15.

TAGAWA, T., and BONNER, J. (1957). Mechanical properties of the *Avena* coleoptiles as related to auxin and to ionic interactions. Ibid. **32**, 207–12.

WOOLLEY, D. W. (1946). Strepogenin activity of seryl-glycylglutamic acid. *J. Biol. Chem.* **166**, 783–4.

The Production of the *Striga* and *Orobanche* Germination Stimulants by Maize Roots

II. CONDITIONS OF SYNTHESIS IN THE ROOT

N. SUNDERLAND

John Innes Horticultural Institution, Bayfordbury, Hertford, Herts.

Received 25 November 1959

SUMMARY

Six serial segments, each 3·0 mm. in length, have been excised from the tip backwards along the maize root and groups of each fragment have been aerated in water and in solutions of sugars for varying times. The amounts of the complex of *Orobanche* and *Striga* germination stimulants which accumulate in the culture fluids have been assessed and compared with the amounts initially present in the tissue at the time of excision from the root.

It is shown that with each fragment more of the complex appears externally when sugar is supplied. 10^{-2} M. solutions of glucose, fructose, or sucrose promote an optimal effect. The amounts in the external solutions are inversely proportional to the initial contents of the segments. The first segment (0–3 mm.) contains the highest proportion of the complex initially, but when immersed in water or the sugar solution very little of it appears externally. The second segment (3–6 mm.) contains the least initial quantity of the complex but in water or sugar produces considerably more than any other segment and more than is originally present. As the series is traversed from segments 2 to 6, the segments have progressively larger initial contents than has segment 2, but after aeration in the sugar solutions the amounts accumulating externally are less than with segment 2 and decrease with their increasing distance from the root tip.

The significance of these observations is discussed.

INTRODUCTION

In the previous paper (Sunderland, 1960) it was shown that a solution of glucose after exposure to fragments of maize roots promotes the germination of seeds of *Orobanche minor* and *Striga hermonthica*. Germination is due, not to glucose, but to a complex of ether-soluble substances which accumulates in the sugar solution. Part of this complex, referred to as the stimulant M_o, germinates seeds of *O. minor*; and part, M_s, germinates seeds of *S. hermonthica*.

The data of the previous paper are confined to a segment of the root excised at a distance of 3–6 mm. from the tip. Evidence is provided which indicates that this segment when immersed in sugar synthesizes part of the stimulant complex. The present series of data provides further evidence for this conclusion and extends the analysis to other zones of the root. Six segments, each 3·0 mm. in length, and excised from the tip backwards along the root have been examined. Groups of fragments have been cultured in water and in solutions of different sugars and sugar derivatives for various times

and the stimulant contents of the resultant fluids have been compared by means of the parasite seeds. Aqueous extracts of the fragments have also been examined to ascertain their initial stimulant contents. Gross differences between solutions were confirmed chromatographically.

MATERIALS AND METHODS

The techniques used in (*a*) the preparation of the segment fluids and aqueous extracts, (*b*) the biological test, and (*c*) the preparation of paper chromatograms were the same as those described in the previous paper.

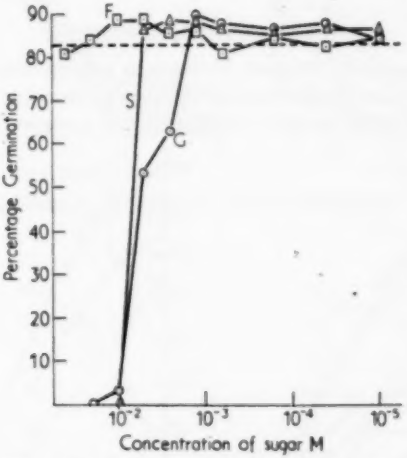


FIG. 1. Effect of glucose G, sucrose S, and fructose F upon germination of *O. minor* in the standard stimulating solution. The response in the standard alone is represented by the dotted line.

Each extract or segment fluid was prepared in triplicate and the three replicates were mixed prior to bioassay. In order to compare readily the stimulant contents of the solutions, 'half-values' were calculated, wherever possible, in the manner described by Brown, Greenwood, Johnson, and Long (1951). These workers found the degree of dilution of the test solutions required to give half the percentage germination promoted by a standard stimulating solution. In the present investigation seed response was often lower than half that given by the standard so that, in some instances, the degree of dilution required to promote a quarter of the standard response was calculated. Differences between experiments due to variability of the parasite seeds were eliminated by adjusting the calculated half-values to a standard value of 25. As a standard the diffusate solution from linseed described by Brown *et al.* (1951) was used.

In a preliminary phase of the investigation effects which the presence of sugars in the stimulating solutions might have on the response of the seeds

were investigated by adding known amounts of sugars to the standard stimulating solution. In one experiment, the results of which are shown in Fig. 1, glucose, sucrose, and fructose were added to aliquots of the standard to give concentrations ranging from 10^{-1} to 10^{-5} M. Concentrations of glucose or sucrose greater than 10^{-2} M. completely suppressed germination of *O. minor*, but fructose did not. The effect was not observed with *S. hermonthica*. Consequently a concentration of 10^{-2} M. sugar was adopted for the experiments so that the inhibitory effect would not interfere with the interpretation of the results. It was found later that the concentration of 10^{-2} M. was, in fact, optimal for the experiments.

EXPERIMENTAL RESULTS

Culture of the segments in water. Percentage germination of seeds of *Orobanche* and *Striga* in solutions obtained from two consecutive 6-hour cultures of segments 1–6 in water is shown in Figs. 2 and 3 respectively. It is evident

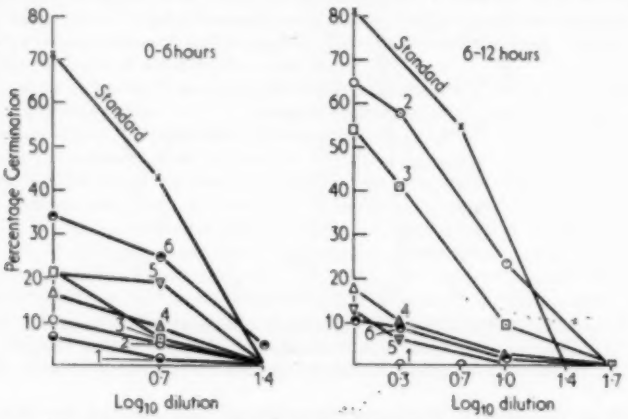


FIG. 2. Mean percentage germination of *O. minor* in the fluids obtained from culture of segments 1–6 in water for two consecutive 6-hour periods at 25° C.

that the responses of the seeds towards the six solutions differ considerably. With *Orobanche* after 0–6 hours, none of the solutions promotes a response as great as that of the standard; the most active preparations being derived from the back part of the root. The greatest response is shown towards the solution from segment 6, the next greatest towards the solution from segment 5, and the response then falls as the series is traversed towards segment 1.

After 6–12 hours it is apparent that the stimulant contents of the solutions again vary but the order of activity is now different. The most active preparation obtained during the second 6 hours is derived from segment 2; the next most active from segment 3. Both these solutions promote responses greater than the corresponding solutions from the first 6 hours. With increasing distance from the root tip the activities of the solutions decrease, and it is

clear that the solutions from segments 5 and 6 are less active after the second than they are after the first 6 hours. With segment 1 the solution after the second 6 hours is less active than it is after the first 6 hours, but for both periods the solutions are less active than any of the others.

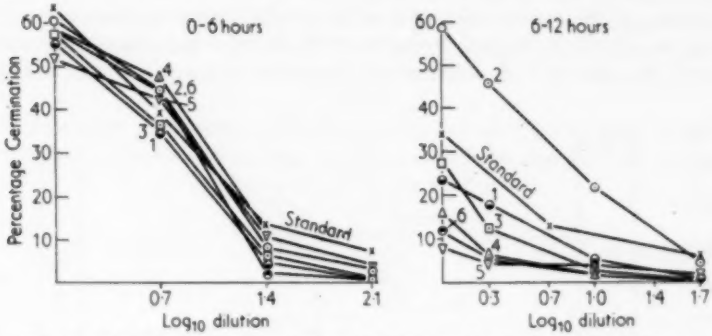


FIG. 3. Mean percentage germination of *S. hermonthica* in the fluids of Fig. 2.

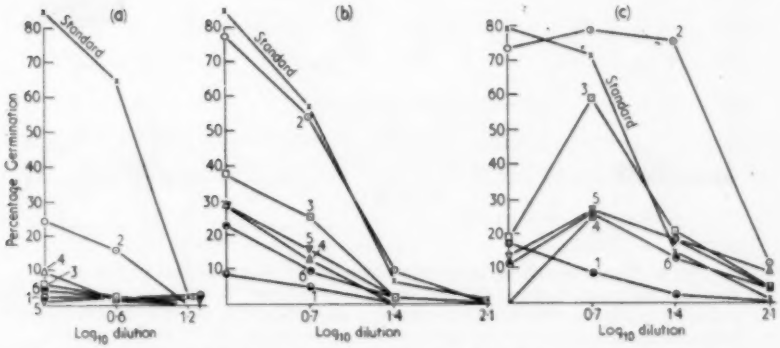


FIG. 4. Mean percentage germination of *O. minor* in the fluids obtained from culture of segments 1–6 in water (*a*), 2×10^{-3} M. glucose (*b*), and 10^{-2} M. glucose (*c*) for 24 hours at 25° C.

A high activity of the fluids from the older parts of the root after only 6 hours in water is also shown by the data for *Striga* seeds (Fig. 3). The most active preparations after 0–6 hours are derived from segments 2, 4, 5, and 6 and the least active from segments 3 and 1. But the difference between these solutions is not very marked; they all promote a response similar in magnitude to that of the standard. After 6–12 hours, however, there is a marked change. Only one solution now promotes a response which exceeds that of the standard. The most active solution is from segment 2, the next most active from segment 1, and the response then falls as the series is traversed to segment 6.

The activity of the solution from segment 2 is greater than it is during 0–6 hours, but the activities of the solutions from all the other segments are less.

After 6–12 hours the data for *Striga* and *Orobanche* are similar except in one respect. With *Orobanche* the solution with the next greatest activity is from segment 3 but with *Striga* it is from segment 1.

Culture of the segments in sugar solutions. Percentage germination of *O. minor* in solutions derived from the culture of segments 1–6 in water, 2×10^{-3} M., and 10^{-2} M. solutions of glucose for 24 hours is shown in Fig. 4.

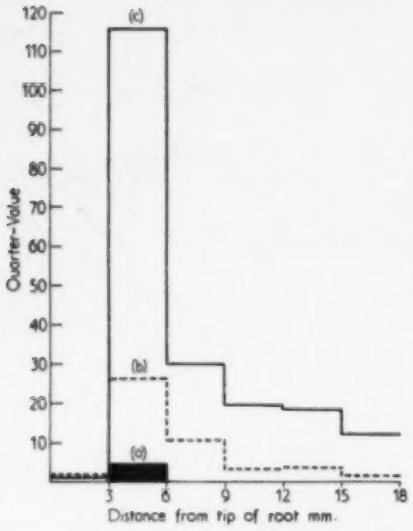


FIG. 5

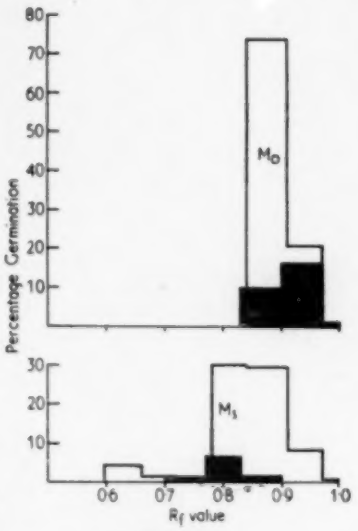


FIG. 6

FIG. 5. Histograms showing quarter-values derived from the data of Fig. 4.
FIG. 6. Histograms showing different amounts of M_0 and M_1 on paper chromatograms of the fluids obtained with segment 2 after culture in 2×10^{-3} M. glucose (solid histogram) and 10^{-2} M. glucose for 24 hours at 25° C. Solvent-tertiary butanol 4 : ethanol 2 : water 2.

'Quarter-values' for most of the solutions are shown in Fig. 5 and the corresponding data for *Striga* are shown in Table I.

It is evident that the order of activity of the solutions is the same after 24 hours whether the tissue is exposed to water or to the glucose solutions. The highest response is promoted by the solution from segment 2, the next highest by the solution from segment 3, and the response falls as the series is traversed to segment 6. The fluid from segment 1 is always the least active preparation. It is also evident that the solutions obtained from cultures in sugar are all more active than the corresponding cultures in water. With segment 2, for example, immersion in the 2×10^{-3} M. solution of glucose raises the quarter-value about 5-fold from 4·7 to 26·6. It is raised a further

5-fold from 26·6 to 116 by increasing the initial concentration of sugar to 10^{-2} M. Clearly more M_o appears externally with the higher concentration of sugar as is indicated by the data of Fig. 6. Increments in the amount of M_o also occur with the other segments when they are immersed in the higher concentration of sugar. The data of Fig. 5 indicate a 5-fold increase in the stimulant contents of the solutions from segments 4, 5, and 6, and about a 3-fold increase with segment 3. The low response of the seeds in the

TABLE I

Mean percentage germination of seeds of Striga hermonthica *in fluids prepared by culture of segments 1–6 in water and in* 2×10^{-3} *M. and* 10^{-2} *M. solutions of glucose at 25° C. for 24 hours*

		Segment						
	Dilution	1	2	3	4	5	6	Standard
Water	1	1·0	1·0	0·9	0·9	—	—	33·2
	½	nil	1·4	nil	nil	—	—	—
Glucose 2×10^{-3} M.	1	nil	9·4	2·1	1·4	2·1	2·9	35·4
	½	nil	nil	nil	nil	nil	nil	—
Glucose 10^{-2} M.	1	14·4	24·3	12·0	7·6	6·0	4·7	24·0
	½	2·1	6·8	3·9	0·9	0·5	1·3	9·6
	1/15	nil	2·0	nil	nil	nil	0·4	2·3
	1/125	nil	0·5	nil	nil	nil	nil	nil

TABLE II

Half-values for fluids derived from the culture of segment 2 (3–6 mm.) in water W, and in 10^{-2} *M. solutions of glucose G, fructose F, and sucrose S at 25° C. for 24 hours*

	Experiment 1			Experiment 2		
	W	G	F	W	G	S
Orobanche	7	160	23	< 1	40	41
Striga	< 1	6·3	5·2	0	< 1	3·3

undiluted solutions from segments 3, 4, 5, and 6 after culture in the higher concentration of sugar is due to inhibition of germination by glucose.

The data for *Striga* (Table I) are similar in most respects to those for *Orobanche*. With each segment the solutions derived from the sugar cultures promote greater responses than those from water cultures and the greatest responses are obtained with the higher concentration of sugar. As with *Orobanche* the fluid from segment 2 promotes the highest response of the seeds and the response decreases with each successive solution. The data of Fig. 6 indicate a higher amount of M_s after culture in the higher concentration of sugar.

The data differ from those for *Orobanche* in that the next greatest response is promoted by the fluid derived, not from segment 3, but from segment 1. This is also the case, it will be recalled, with the data of Fig. 3 during 6–12

hours. A further feature of the data for *Striga* is the difference in the activities of the solutions after 24 hours' culture and after only 6 hours in water (Fig. 3). The activities after the longer period of culture are less than after the shorter period. This is probably due, as suggested in the previous paper, to instability of the stimulant M_s in water.

Results similar to those obtained with glucose are also given by sucrose and fructose. The data for segment 2 are shown in Table II. The effect of culturing this segment in solutions of fructose and sucrose is compared with cultures in water and glucose. The half-values indicate that after 24 hours glucose,

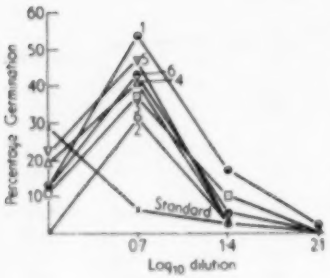


FIG. 7

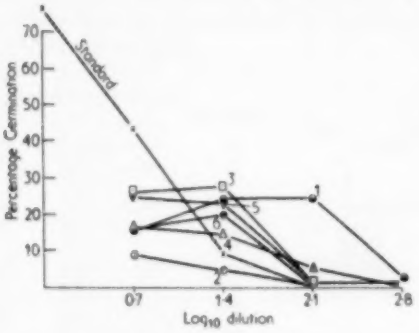


FIG. 8

FIG. 7. Mean percentage germination of *S. hermonthica* in aqueous extracts of segments 1–6 (20 segments per ml.).

FIG. 8. Mean percentage germination of *O. minor* in the aqueous extracts of Fig. 7 after dilution with the standard stimulating solution ($\frac{1}{100}$) instead of water.

sucrose, and fructose give more active preparations than does water; but the resulting fluids are clearly not identical in activity. Commencing with a concentration of 0·01 M., fructose yields less active preparations in 24 hours than does glucose, and it is probable that sucrose yields slightly more active preparations than glucose.

Aqueous extracts. The initial contents of stimulants in the segments immediately after their excision from the root are indicated for *Striga* by the data of Fig. 7. The undiluted extracts are inhibitory but from the data obtained with the diluted extracts it is clear that the solution promoting the highest response and hence containing the highest amount of M_s is obtained from segment 1. The response of the seeds then falls markedly in the extract from segment 2, and rises as the series is traversed to segment 6. It is particularly significant that the least active extract is derived from segment 2 although it gives rise to the most active segment fluids.

The aqueous extracts, even when diluted, at least with water, do not germinate seeds of *O. minor* because of the presence of glucose and a low content of M_o. In the previous paper it is shown that the response of the parasite seeds is considerably enhanced by mixing M_o with a trace of the stimulant

from linseed roots. The amounts of M_o in the extracts have therefore been compared, by diluting them with a solution containing a trace of the linseed stimulant instead of with water. The data are shown in Fig. 8. There is much inhibition in the undiluted solutions due to glucose, but from the data obtained with the diluted extracts it is evident that the initial contents of M_o in the segments follow a similar order to those of M_s. The responses in the extracts from segments 3, 4, 5, and 6 fall intermediate between those from segments 1 and 2. The highest initial content is in segment 1 and the least is in segment 2.

DISCUSSION

The results obtained by immersing the root fragments in water for short periods of time are of considerable importance for the interpretation of the group of observations presented above. The appearance of stimulating substances in the water cannot be ascribed simply to the diffusion outwards of the

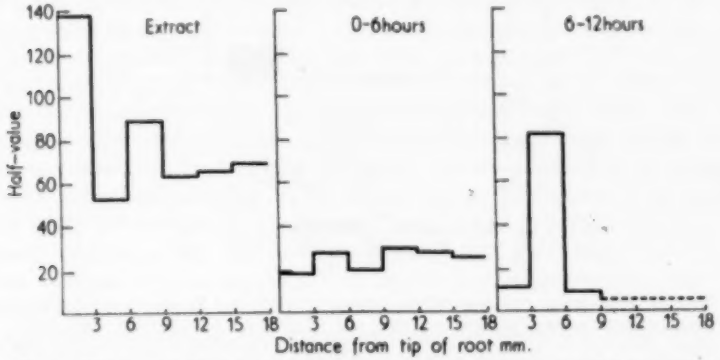


FIG. 9. Histograms showing half-values of the extracts of Fig. 7 and the segment fluids of Fig. 3.

residual stimulants from the fragments. This is most readily seen by comparing the amount of stimulant which accumulates externally in water with the initial content of each fragment.

The relevant data for *Striga* are assembled in Fig. 9. Here half-values calculated from the data of Figs. 3 and 7 are given. It will be observed that in one case, namely segment 2, the total amount of M_s formed externally exceeds that initially present. This means that some M_s is synthesized by the isolated root fragment when it is immersed in water, and since the solution from the second 6 hours is more active than that from the first, it may be concluded that the newly synthesized material probably appears during 6–12 hours.

The behaviour of segment 1 differs markedly from that of segment 2. There is a very large difference between the internal and external amounts of M_s. The initial content of the fragment is high. It has been found, however, that although little of this residual stimulant appears externally, the content of the fragment falls almost to nothing at the end of 12 hours in water (Fig. 10).

This suggests that the failure of M_s to diffuse rapidly from the fragment in water is due to its utilization by the tissue, and no more M_s is being synthesized by the fragment to maintain the high internal content. Thus the data suggest with segment 1 a high rate of utilization and a low rate of synthesis of M_s, whilst with segment 2 there is a high rate of synthesis and a low rate of utilization at least during the 6–12 hour period.

The data obtained with segments 3, 4, 5, and 6 also support the view that the amount of stimulant accumulating externally is determined by the balance

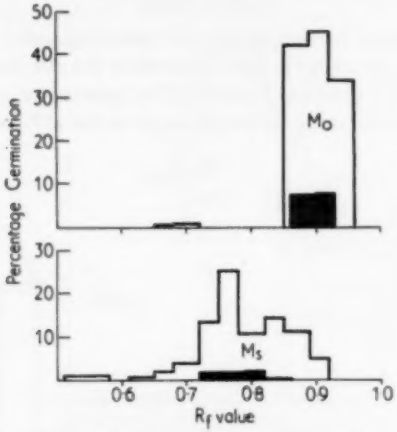


FIG. 10. Histograms showing different amounts of M_o and M_s on paper chromatograms of ether extracts of segment 1 (0–3 mm.) before and after culture (solid histogram) for 12 hours in water at 25° C. Eluted with the standard stimulating solution ($\frac{1}{100}$). Solvent-tertiary butanol 4 : ethanol 2 : water 2.

between the rates at which they are synthesized and consumed by the isolated tissue. It will be observed from Fig. 9 that the total amounts of M_s formed during 12 hours in water by segments 3, 4, 5, and 6 are less than the amounts initially present. Further, the external amounts fall with time. This suggests that the rates of synthesis and utilization are comparatively low. Since the rate of utilization is low, the residual stimulants escape rapidly into water during the first 6 hours, but with continued culture the amounts decrease because little or no M_s is synthesized by the fragments.

The conclusions with respect to M_s also apply to the *Orobanche* stimulant M_o. Evidence that synthesis of M_o occurs when segment 2 is immersed in a glucose solution has already been presented in the previous paper. The present series of data indicates that synthesis also occurs when it is immersed in water. From the data of Fig. 8 it is evident that segment 2 contains less M_o than any other segment, but in water (Fig. 2) a considerable quantity is formed exceeding that formed by the other segments during 6–12 hours. Thus segment 2 has a high rate of synthesis and probably a low rate of consumption.

This also applies to a lesser degree with segment 3. Segment 1, on the other hand, has the greatest initial content of M_0 (Fig. 8), but in water little of this appears externally (Fig. 2). The high level of M_0 internally falls after 12 hours in water (Fig. 10) so that the data are consistent with the view that there is a high rate of consumption of M_0 in segment 1 and a low rate of synthesis. The fragments from the more mature part of the root have initial contents of M_0 greater than that of segment 2, but in water much less is formed externally than with segment 2 and the rate at which M_0 appears externally falls with time. Thus the rates of synthesis and utilization of M_0 are both low with segments 4, 5, and 6.

It is clear from the above considerations that utilization and synthesis are higher in the first six mm. of the root. Segment 1, which consists of cap and dividing cells and part of the elongating zone, increases in length during culture. In a 10^{-2} M. solution of glucose, for example, its length is doubled within 24 hours and it continues to extend if culture is prolonged. Segment 2, on the other hand, which consists of cells undergoing and completing growth in length, increases from 3·0 to 4·0 mm. only and with prolonged culture there is no further increase in length. Utilization of the stimulating substances is greatest during culture when the tissue is increasing in length. When extension ceases in segment 2, synthesis is apparently high. It is suggested therefore that the stimulating substances are utilized in some way during extension growth, a suggestion supported by the fact that in the intact root the level of stimulants is lowest in the region of cell elongation. It is also pertinent that Brown, Robinson, and Johnson (1949) found that the stimulating solution from sorghum roots promotes growth of isolated fragments taken from the elongating zone of pea roots.

The data obtained by immersing the fragments of root in sugar solutions indicate that synthesis of the complex of stimulating substances is dependent upon a supply of sugar. The high level of synthesis of M_0 by segment 2 in water is not maintained beyond 12 hours. Synthesis only continues if the segment is transferred to a sugar solution. In water therefore synthesis occurs owing to utilization of the residual glucose, but when this is exhausted synthesis can no longer proceed. Synthesis by segment 2 has been demonstrated for at least three consecutive 24-hour cultures in a solution of glucose or sucrose. With sucrose, no doubt, it is hydrolysed into glucose and fructose; the monosaccharides are then absorbed by the tissue, and the newly synthesized stimulating materials pass out into the culture medium.

Two possibilities may be considered with regard to the effect of the sugar. It may accelerate metabolic processes on which the formation of the complex depends or the sugar itself may be modified directly by appropriate enzyme systems to yield the active materials. A number of other sugars and sugar derivatives were tried with segment 2 but none of them caused synthesis. These included maltose, lactose, cellobiose, glucose 1-phosphate, *d*-galactose, *d*-mannose, *d*-ribose, *d*- and *l*-arabinose, *d*-xylose, *d*-sorbose, *l*-rhamnose, erythrulose, α-methyl-*d*-glucoside, 2-ketogluconic acid, *d*-xyloketose, malic

and succinic acids. Thus the available evidence is not sufficiently extensive to provide the basis for a choice between the two alternatives.

The zones of the root in which maximal synthesis of M_s and M_o occurs do not absolutely coincide. The most active segment fluids for *Orobanche* are derived from segments 2 and 3, but with *Striga* they are derived from segments 2 and 1. This suggests that the two stimulants are synthesized by different enzyme systems which exhibit their maximal activities at slightly differing regions along the root. It is probable that either glucose or fructose can act as substrate for each enzyme system, but in this respect it is significant that segment fluids prepared from fructose are less active after 24 hours than those prepared with glucose or sucrose (Table II). Preliminary chromatographic data suggest that this difference may be due to different proportions of the constituents of the stimulant complex, but the reason for this is obscure.

To summarize, the data obtained with isolated fragments of root indicate that synthesis of the *Orobanche* and *Striga* germination stimulants occurs in the root of the intact plant and that sugars are involved in their formation. Synthesis occurs in a comparatively short zone just as the cells are completing growth in length. With further development the synthetic capacity of the root cells decreases. The stimulants are utilized during the growth in length of the root cells, and consumption also falls as the cells mature. Thus in intact roots stimulants accumulate in the more mature parts of the root from which they probably escape in greatest quantities into the soil. This is consistent with the findings of earlier workers that the stimulating substances are released from the root-hair zone (Saunders, 1933; Chabrolin, 1939).

LITERATURE CITED

Brown, R., Greenwood, A. D., Johnson, A. W., and Long, A. G. (1951). The stimulant involved in the germination of *Orobanche minor* Sm. I. Assay technique and bulk preparation of the stimulant. *Biochem. J.* **48,** 559.

—— Robinson, E., and Johnson, A. W. (1949). The effects of *d*-xyloketose and certain root exudates in extension growth. *Proc. Roy. Soc.* **B. 136,** 577.

Chabrolin, C. (1939). Contribution à l'étude de la germination des graines de l'orobanche de la Fève. *Ann. Serv. bot. Tunis,* **14–15,** 92.

Saunders, A. R. (1933). Studies in phanerogamic parasitism with particular reference to *Striga lutea*. *Science Bulletin Dept. Agric. S. Africa,* No. 128.

Sunderland, N. (1960). The production of the *Striga* and *Orobanche* germination stimulants by maize roots. I. The number and variety of stimulants. *J. Exp. Bot.* **11,** 236–45.

Interaction of Mineral Deficiency and Nitrogen Source on Acid Phosphatase Activity in Leaf Extracts[1]

E. J. HEWITT AND P. TATHAM[2]

Long Ashton Research Station, University of Bristol

Received 26 January 1960

SUMMARY

Plants were grown in sand cultures to observe the effects of mineral deficiencies, with different sources of nitrogen supply, on leaf acid phosphatase activity using phenolphthalein phosphate as substrate.

Large increases ranging from 7 to 18 times normal (on fresh weight or protein basis) occurred with deficiency of phosphorus with nitrate, nitrite or ammonia nitrogen; increases from 4 to 8 times normal occurred with deficiencies of zinc with nitrite or ammonia; and of copper with nitrite. Less pronounced increases, from 2 to 3 times normal on a protein basis, occurred with deficiency of zinc with nitrate; of boron with all nitrogen sources; of manganese and of iron with nitrite. Activities on both protein and fresh-weight basis were often greater with nitrite or ammonium sulphate than with nitrate. Effects of phosphorus, zinc, copper, boron, and nitrogen sources were significant.

Molybdenum deficiency caused approximately 2 times normal activity on a protein basis in tomato, tobacco, sunflower, cauliflower, lucerne, mustard, spinach beet, and lettuce when nitrate was given. Effects with ammonia nitrogen were not consistent in molybdenum-deficient plants but 50 per cent. increases occurred in the first 5 species listed. Activities varied widely between species.

It was concluded that the increases in activity were caused by increases in amounts of the enzyme produced during growth, and that different combinations of deficiencies and nitrogen supplies produce different specific responses in terms of enzyme synthesis.

INTRODUCTION

MINERAL deficiencies may cause profound changes in the general pattern of enzyme activity in plants even when the deficient element is not directly involved in the activation of some of the enzymes concerned. This aspect of mineral nutrition, to which attention was directed particularly by Nason, Oldewurtel, and Propst (1952), has been reviewed recently (Hewitt, 1958, 1959). Such changes in enzyme pattern may account for some of the unfavourable effects of mineral deficiencies that are distinct from their direct effects. Spencer (1954) drew attention to molybdate as an inhibitor of acid phosphatase activity in tomato plants *in vivo* and *in vitro*. He suggested that

[1] This work was undertaken during the tenure of a Colonial Office Training Studentship by P. Tatham.

[2] Present Address: West African Institute for Oil Palm Research, Benin City, Western Nigeria.

this effect might account for the apparent molybdenum requirement of plants grown with nitrogen sources, such as ammonium compounds, that do not depend on the molybdenum-containing nitrate reductase system (Nicholas and Nason, 1955) for their assimilation. Acid phosphatase activity was demonstrated histologically by Reed (1946) to be high in zinc-deficient tomato plants; some evidence of enhanced acid phosphatase activity was also obtained with boron deficiency (Reed, 1947). To find how general are these effects of mineral deficiencies on acid phosphatase their magnitude has been studied in relation to deficiencies of nine elements in tomato. Because of recent evidence (Nicholas, 1957; Nicholas and Goodman, 1958) that iron, manganese, copper, and zinc are involved in the activity of enzymes concerned in stages of inorganic nitrogen metabolism in fungi, various sources of nitrogen were used to determine whether interactions occurred between micronutrients and nitrogen supplies in higher plants, and whether these produced any secondary effects on acid phosphatase activity. Several species were tested with molybdenum deficiency to find if any relationship existed between apparent molybdenum requirements with ammonium sulphate and acid phosphatase activity.

MATERIALS AND METHODS

Plant cultures. Plants were grown in sand cultures using the methods for sand, water, and nutrient reagent purification which have been described elsewhere (Hewitt, 1952). Calcium carbonate was mixed in the sand in all treatments at the rate of 1·5 g. per kilo of sand as described by Agarwala and Hewitt (1955) to prevent injury when ammonium sulphate was given. For molybdenum deficiency, calcium carbonate was prepared by passing carbon dioxide into a solution of purified calcium chloride (Hewitt, 1952, 1955) in the presence of redistilled ammonia. For the other deficiencies, analytical reagent grade calcium carbonate was used.

The nutrient solutions were based on the Long Ashton formula (Hewitt, 1952) but calcium chloride and potassium sulphate were used instead of nitrates in all nitrogen treatments. Nitrogen was given as sodium nitrate, or nitrite, or as ammonium sulphate. The nitrogen level in all treatments was reduced from 12 to 6 mM./L. after 4 weeks in the first experiment to eliminate toxic effects of nitrite, and these levels were used throughout in the two later experiments. In the first experiment on macro-nutrient deficiencies, 4 litres of full nutrient were given for 2 weeks before commencing the deficiency treatments. In the second experiment in the respective deficiency treatments boron was given at one-twentieth and other micronutrients at one-fiftieth the normal level. In the third experiment, molybdenum was omitted completely from the deficiency treatment.

Acid phosphatase determination. Plants were sampled when 7 weeks old in May in the first experiment, when 9 weeks old in July in the second experiment, and when 6 weeks old in August in the third experiment. Symptoms of the deficiencies were clearly evident in all experiments at the time of sampling. Assays were made on 4 separate replicates from randomized blocks

in the first and second experiments and single estimations were made on 8 different species in the third experiment (on molybdenum deficiency). The enzyme assays were made on sub-samples of 2·0 g. of freshly harvested leaf lamina. This was taken from thoroughly mixed, finely chopped leaf tissues representing the whole foliage of one plant after removal of the midrib and all necrotic or wilted portions of lamina. Immediately after weighing, the samples were thoroughly ground at 0° C. in a chilled mortar with acid-washed sand and 10 ml. of 0·1 N. sodium succinate buffer, pH 6·0 (Spencer, 1954). After centrifuging, the extracts could be stored in ice for at least 4 hours without appreciable loss of activity; this allowed one set of replicates from each group of deficiency treatments to be examined at one time. Further sets were assayed on succeeding days. Since dialysis for 22 hours at 4° C. against succinate buffer caused no gain or loss in activity, this procedure was omitted from preparation of the extracts.

The assay system comprised 3 ml. of 0·2 M. sodium acetate-acetic acid buffer; pH 5·4, 1 ml. 0·00624 M. (125 mg. in 50 ml.) phenolphthalein phosphate, 3·8 ml. water, and 0·2 ml. extract. Preliminary tests showed that non-enzymic hydrolysis was negligible during several hours at 27° C. The blanks were therefore made up with 4·8 ml. water and no substrate, but included the extract to allow for the effects of alkali on colour or turbidity. The reaction was terminated after incubation for 20 minutes at 27° C. by the addition of 1 ml. of 15 per cent. trichloracetic acid, followed by 1 ml. of N. sodium hydroxide, which brought the pH to 9·8–10·5. The light absorption of liberated phenolphthalein was measured on a Unicam SP 600 spectrophotometer at 550 mμ in a 1 cm. cell within 10 minutes of producing the colour, and the amount of hydrolysis was calculated from a calibration curve prepared with pure phenolphthalein over the range 0–35 μM. in a volume of 10 ml.

Protein determination. Protein in the extracts was estimated by the Folin method (Lowry *et al.*, 1951). Protein was precipitated by adding 1 ml. of 15 per cent. trichloracetic acid to 1 ml. of extract, making up to 10 ml. with water and centrifuging. The supernatant fluid was decanted and the protein was dissolved in 0·5 ml. N. sodium hydroxide, at 80° C., 9·4 ml. water was added and an aliquot of 0·7 ml. was taken for protein estimation, using pure casein as a standard. The method has been found to compare satisfactorily with the estimation of casein and leaf-extract proteins by micro-Kjeldahl methods.

RESULTS

Leaf Symptoms. The symptoms of mineral deficiencies in tomato grown with nitrate have been described elsewhere (Hewitt and Bolle-Jones, 1950, 1952; Wallace, 1951). When nitrate was replaced by nitrite or ammonia the symptoms were not notably changed, except with deficiencies of copper and molybdenum.

With copper deficiency, plants grown with nitrite or ammonium sulphate were chlorotic, far more stunted in growth, and lacked the characteristic

blue-green appearance produced with nitrate. All species grown in the nitrate treatment showed typical moderate to severe molybdenum deficiency symptoms and cauliflower given ammonium sulphate developed whiptail symptoms (Agarwala and Hewitt, 1955) as expected. Tomato developed a bright yellow-green interveinal mottling of upper leaves when ammonium sulphate was given and similar symptoms have been observed in separate experiments with A. J. Abbott. The other plants showed no leaf symptoms of molybdenum deficiency in the presence of ammonia nitrogen except lettuce, which was chlorotic. Vigour was notably decreased in mustard and tobacco but not in beet, lucerne, or sunflower under these conditions.

Acid phosphatase activity. Detailed results are set out in Tables I–III. On a

TABLE I

Effects of macronutrient deficiencies on acid phosphatase activity in tomato leaf tissues

Nitrogen source	Control	−Mg	−K	−P	Means
	(*i*) *on fresh-wt. basis**				
NO_3	8·80	8·38	13·25	61·30	22·93
NO_2	8·93	8·13	13·43	83·38	28·46
NH_4	9·33	7·25	12·95	105·28	33·70
Means	9·02	7·92	13·21	83·32	—
LSD Separate treatments	5% 16·15	Nitrogen sources (Means)		5%	8·08
LSD " "	0·1% 28·71	" "	"	1%	10·85
	(*ii*) *on protein basis*†				
NO_3	0·234	0·293	0·452	2·177	0·789
NO_2	0·221	0·221	0·404	2·688	0·884
NH_4	0·230	0·256	0·367	4·198	1·263
Means	0·228	0·257	0·408	3·021	—
LSD Separate treatments	5% 0·666	Nitrogen sources (means)		5%	0·333
LSD " "	0·1% 1·222	" "	"	1%	0·453

'Soluble' protein mg. per g. leaf

Means for NO_3	13·43	LSD 5%	1·08
" " NO_2	15·50	LSD 1%	2·00
" " NH_4	14·59		
Means for control	17·22	LSD 0·1%	2·30
" " −Mg	13·67		
" " −K	13·72		
" " −P	13·42		

* μM. phenolphthalein phosphate hydrolysed per 100 mg. tissue in 20 min.
† μM. " " " " 1 mg. protein per min.

fresh-weight basis, acid phosphatase activities were outstandingly higher than complete nutrient treatments for phosphorus deficiency regardless of nitrogen source ($P < 0{\cdot}001$), for zinc deficiency in the presence of nitrite or ammonia nitrogen ($P < 0{\cdot}001$), and for copper deficiency in the presence of

TABLE II

Effects of micronutrient deficiencies on acid phosphatase activity in tomato leaf tissues

Nitrogen source	Control	−B	−Fe	−Mn	−Cu	−Zn	Means
			(i) on fresh-wt. basis•				
NO_3	8·53	9·45	8·73	10·58	11·93	11·65	10·14
NO_2	9·10	10·70	14·20	17·00	62·78	52·63	27·73
NH_4	9·38	13·78	9·60	12·50	13·15	34·68	15·51
Means	9·00	11·31	10·84	13·36	29·28	32·98	—

LSD	Separate treatments	5%	10·74	Nitrogen sources (Means)		5%	4·38
LSD	,, ,,	1%	14·33	,, ,,	,,	1%	5·85
LSD	,, ,,	0·1%	18·72	,, ,,	,,	0·1%	7·64

			(ii) on protein basis†				
NO_3	0·221	0·492	0·223	0·243	0·381	0·458	0·336
NO_2	0·226	0·551	0·347	0·478	1·626	1·662	0·815
NH_4	0·213	0·612	0·243	0·312	0·322	0·899	0·434
Means	0·220	0·552	0·271	0·345	0·776	1·006	—

LSD	5%	Separate treatments	0·337	Means of treatments	0·195	Means of Nitrogen sources	0·138
LSD	1%	,,	0·450	,,	0·260	,, ,,	0·184
LSD	0·1%	,,	0·588	,,	0·339	,, ,,	0·240

'Soluble' protein mg. per g. leaf

Means for NO_3	15·78	LSD 5%	1·37
,, ,, NO_2	16·44	LSD 1%	1·83
,, ,, NH_4	18·11	LSD 0·1%	2·38
Means for control	19·71	LSD 5%	1·94
,, ,, −B	9·55	LSD 1%	2·54
,, ,, −Fe	19·20	LSD 0·1%	3·37
,, ,, −Mn	18·72		
,, ,, −Cu	18·31		
,, ,, −Zn	15·25		

• μM. phenolphthalein phosphate hydrolysed per 100 mg. tissue in 20 min.
† μM. ,, ,, ,, ,, 1 mg. protein per min.

nitrite ($P < 0.001$). Slightly but not significantly increased activities were produced in some other deficiencies, namely: for potassium with all sources of nitrogen; boron with ammonia nitrogen; iron with nitrite; manganese with nitrite and ammonia; copper with nitrate and ammonia; zinc with nitrate. Similar and often proportionately greater increases were apparent for specific activities on a protein basis with identical degrees of significance. Activities also appeared to be increased (at the threshold of significance P: = 0·05–0·10), for boron deficiency with nitrite and ammonium sulphate and the difference for boron between means for all nitrogen treatments was fully significant ($P < 0.001$).

There were also significant effects of nitrogen treatments. Thus for zinc deficiency, acid phosphatase activities were increased in the order $NO_3 < NH_4$ ($P < 0.05$ protein basis, $P < 0.001$ fresh-wt. basis) $< NO_2$ ($P < 0.001$

TABLE III

Effects of molybdenum deficiency on acid phosphatase activity and 'soluble' protein in leaves of different species*

Plant	Treatment	Acid Phosphatase Activity*		Protein mg./g.
		per 100 mg. fresh wt.	per mg. protein	
Tomato	N as NO_3 + Mo	14·1	1·44	4·9
	− Mo	7·5	2·83	1·3
	N as NH_4 + Mo	9·9	1·37	3·6
	− Mo	16·8	1·72	4·8
Tobacco	N as NO_3 + Mo	6·0	1·00	3·0
	− Mo	3·6	2·34	0·8
	N as NH_4 + Mo	3·6	0·75	2·4
	− Mo	10·8	1·80	3·0
Cauliflower	N as NO_3 + Mo	120·3	10·32	5·8
	− Mo	45·6	22·31	1·0
	N as NH_4 + Mo	65·7	7·97	4·1
	− Mo	123·9	17·24	3·6
Mustard	N as NO_3 + Mo	56·4	1·71	16·5
	− Mo	64·2	3·18	10·1
	N as NH_4 + Mo	193·5	7·45	13·0
	− Mo	141·0	4·51	15·6
Lettuce	N as NO_3 + Mo	8·4	0·41	10·3
	− Mo	8·4	0·78	5·4
	N as NH_4 + Mo	15·0	0·94	8·0
	− Mo	18·9	0·92	10·3
Sunflower	N as NO_3 + Mo	100·8	4·93	10·2
	− Mo	184·8	10·34	8·9
	N as NH_4 + Mo	273·9	7·52	18·2
	− Mo	290·7	10·22	14·3
Spinach beet	N as NO_3 + Mo	26·1	1·65	7·9
	− Mo	12·4	2·21	2·8
	N as NH_4 + Mo	21·6	1·02	10·6
	− Mo	18·9	0·92	10·3
Lucerne	N as NO_3 + Mo	174·9	7·10	11·0
	− Mo	224·4	15·82	7·1
	N as NH_4 + Mo	157·8	9·41	8·4
	− Mo	360·3	14·13	12·8
Mean of NO_3 series	+ Mo	63·4	3·57	—
	− Mo	68·9	7·48	—
„ „ NH_4 series	+ Mo	93·1	4·50	—
	− Mo	122·7	6·43	—

* Activities as μM. phenolphthalein phosphate hydrolysed per 100 mg. fresh wt. in 20 min. or per mg. protein per min.

protein basis, $P < 0.01$ fresh-wt. basis). In phosphorus-deficient plants, the activities with ammonium sulphate were significantly greater ($P < 0.01$) than with nitrite or nitrate in decreasing order of activity on fresh-weight basis, and in the same order ($P < 0.01$ for each difference) on protein basis of specific activity. The effect of nitrite on the activity in copper-deficient plants was outstanding ($P < 0.001$). Nitrite also appeared to enhance activities observed for other micronutrient treatments in the second experiment. Individual differences were not significant but the overall effect of nitrite in producing

greater activities compared with nitrate or ammonium sulphate was highly significant ($P < 0.001$). In the first experiment with macronutrients, activities were significantly greater with ammonium sulphate ($P < 0.001$) than with nitrite and nitrate, which did not show significantly different means.

In the third experiment, with single estimations, values varied widely between different species—being lowest for lettuce and highest for cauliflower—but were characteristically grouped with respect to each species. On a protein basis, where values extended over a 50-fold range, activities were greater for molybdenum deficiency without exception with nitrate; effects were not consistent with ammonia, but large increases occurred in some species. On a fresh-weight basis the effects of molybdenum deficiency were not consistent, but activities were consistently higher with ammonia than with nitrate in the molybdenum-deficient plants.

Protein. The protein remaining in the supernatant solution containing the enzyme was decreased in a highly significant manner ($P < 0.001$) by deficiencies of boron, zinc, magnesium, potassium, and phosphorus, but not by deficiencies of iron, manganese, or copper. Mean values were significantly higher with ammonium sulphate than with nitrate ($P < 0.05$ and 0.01 respectively) in the first and second experiments. Nitrite produced significantly higher amounts than nitrate in the first experiment ($P < 0.001$) but not in the second.

In the third experiment, molybdenum deficiency consistently decreased the protein extracted from plants grown with nitrate, and deficient plants grown with ammonium sulphate yielded more protein than those grown with nitrate. The amounts of protein extracted from different species (Table III) varied widely but appeared to be characteristic for each. The amounts extracted from tomato in the third experiment were comparatively low, possibly due to effects of age, season, or other factors.

DISCUSSION

The possible ways in which the observed differences in values for acid phosphatase activity and 'soluble' protein might have arisen may be considered. Previous experiments with cauliflower (Candela *et al.*, 1957) showed that the acid phosphatase and 'soluble' protein showed comparable behaviour with regard to their degree of extraction from different leaves, and it is assumed for the following discussion that *extent* of extraction of protein and of acid phosphatase from the plants used in the present work occurred proportionally regardless of treatments. It is conceivable that the degree of protein extraction achieved differed with respect to species, but this would not affect arguments based on the assumption stated above. Different treatments might result in different degrees of extraction, owing to different degrees of cell rupture or of protein denaturation to an insoluble state, thereby affecting activities on a fresh-weight but not on a protein basis. With respect to deficiencies of potassium, phosphorus, boron, and molybdenum, however, the effects recorded here of these treatments on 'soluble' protein are wholly consistent with the

effects of these deficiencies on total protein content in plants reported in other work (Richards, 1938; Gauch and Dugger, 1954; Hewitt, Agarwala, and Williams, 1957). There is therefore no reason at present to doubt that marked decreases in 'soluble' protein, where recorded for different treatments, reflect real decreases in the cell content of protein. The changes in acid phosphatase activities observed here are therefore considered to be due either to changes in amounts of naturally present inhibitors or to the amounts of enzyme present in the tissues relative to fresh weight or to cell protein.

Cell contents were diluted about 230 times before assay and differential effects of dissociable inhibitors would be expected to become small. Dialysis of the extracts, tested initially, never resulted in greater activities. The presence of dialysable inhibitors was therefore discounted as a general explanation.

With special reference to possible effects of molybdate (Spencer, 1954), it can be calculated from known concentrations of molybdenum in plant material that the concentrations present in the assay systems would be about 3×10^{-8} M. and 2×10^{-10} M. for full nutrient and molybdenum-deficient plants respectively. These concentrations as molybdate are far below the thresholds for inhibition of acid phosphatase *in vivo* or *in vitro* recorded by Spencer, and it would therefore appear unlikely that the effects of molybdenum treatments, which were often large, and of the other deficiencies tested in our work were due to the levels of molybdate present in the assay systems.

The discovery by Kern and Natale (1958) that *Mycobacterium butyricum* contains a non-dialysable protein inhibitor of the DPN-ase enzyme present in this organism, although it does not inhibit DPN-ase from *Neurospora crassa* which apparently has no such inhibitors (Nason, Kaplan, and Colowick, 1951), suggested that effects of such factors should be considered as possible explanations of the present results. In separate experiments with phosphorus-deficient and normal plants of the same age showing large differences in acid phosphatase activity there was good arithmetic agreement between activities determined in assays on separate and combined extracts from the two treatments in different proportions. Normal plants do not, therefore, appear to contain dissociable non-dialysable inhibitors which are absent from plants showing a high activity. Phosphorus-deficient plants do not contain corresponding activators of the acid phosphatase system.

We conclude from this discussion that the observed effects of certain deficiency treatments on acid phosphatase activity were produced during the growth of the plants and most probably resulted from increased synthesis of the enzyme protein during growth, especially in relation to the production of protein as a whole. Nitrite as source of nitrogen was especially notable in increasing acid phosphatase with some deficiencies though this effect is, as yet, unexplained. Nitrogen supply had large effects on the activity of DPN-ase in *Neurospora* where decreased nitrogen level, or nitrogen given as nitrite, produced high activities (Nason, Kaplan, and Oldewurtel, 1953).

Torriani (1959) reported that not only is alkaline phosphatase activity in

E. coli inhibited by free inorganic phosphate but that synthesis of the enzyme is inhibited by phosphate and can only occur when the inorganic phosphate concentration becomes relatively low. In our work it may be noted, however, that although phosphorus deficiency leads to a low inorganic phosphate level (unpublished data) zinc and molybdenum deficiencies have the reverse effect (Reed, 1946; Hewitt, 1959).

With regard to the suggestion (Spencer, 1954) that inhibition of acid phosphatase by molybdate may explain molybdenum requirements for plants grown with ammonium sulphate (Agarwala and Hewitt, 1955; Hewitt and McCready, 1956), the results obtained here (Table III) show that the largest increase in activity on protein basis caused by molybdenum deficiency occurred in cauliflower, which is the most susceptible species under these conditions. There was, however, a large increase in the activity in molybdenum-deficient lucerne, especially on a fresh-weight basis, but this plant showed no response whatsoever to the omission of molybdenum in the presence of ammonium sulphate. In general, there was no obvious relationship between vigour or symptoms under these conditions and acid phosphatase activity. Furthermore, infiltration of cauliflower leaves by molybdate has not caused decreases in acid phosphatase activity (Candela, Fisher, and Hewitt, 1957).

It is clear from our results and work reviewed elsewhere (Hewitt, 1958, 1959) that, although enhanced activities of certain enzymes may occur under many different circumstances of nutritional deficiency, such effects may be of specific rather than of general occurrence with respect both to the deficiencies involved and to the enzyme systems affected.

ACKNOWLEDGEMENT

The statistical analysis was carried out by Mr. G. M. Clarke, to whom we are most grateful.

LITERATURE CITED

AGARWALA, S. C., and HEWITT, E. J. (1955). Molybdenum as a plant nutrient VI. The effect of molybdenum supply on the growth and composition of cauliflower plants given different sources of nitrogen supply in sand culture. *J. Hort. Sci.* **30**, 163–80.

CANDELA, M. I., FISHER, E. G., and HEWITT, E. J. (1957). Molybdenum as a plant nutrient X. Some factors affecting the activity of nitrate reductase in cauliflower plants grown with different nitrogen sources and molybdenum levels in sand culture. *Plant Physiol.* **32**, 280–8.

GAUCH, H. G., and DUGGER, W. M., JR. (1954). *The physiological action of boron in higher plants: a review and interpretation.* University of Maryland Agr. Exp. Sta. Bull. A—80 (Technical).

HEWITT, E. J. (1952). *Sand and water culture methods used in the study of plant nutrition.* Tech. Comm. No. 22 Commonwealth Bureau of Horticulture, East Malling, England.

—— (1958). The role of mineral elements in the activity of plant enzyme systems. *Encyclopaedia of Plant Physiology* **4**, 427–81. (Ed. W. Ruhland, Springer, Berlin.)

—— (1959). The metabolism of micronutrient elements in plants. *Biol. Revs. Cambridge Phil. Soc.* **34**, 333–77.

HEWITT, E. J., AGARWALA, S. C., and WILLIAMS, A. H. (1957). Molybdenum as a plant nutrient VIII. The effect of different molybdenum levels and nitrogen supplies on the nitrogen fractions in cauliflower. *J. Hort. Sci.* **32,** 34–48.

—— and BOLLE-JONES, E. W. (1950). The effect of zinc and copper deficiencies on crop plants grown in sand culture. *Long Ashton Res. Sta. Ann. Rep.*, 1950, 56–63.

—— —— (1952). Molybdenum as a plant nutrient II. The effects of molybdenum deficiency on some horticultural and agricultural crops grown in sand culture. *J. Hort. Sci.* **27,** 257–65.

—— and MCCREADY, C. C. (1956). Molybdenum as a plant nutrient VII. The effects of different molybdenum and nitrogen supplies on yields and composition of tomato plants grown in sand culture. Ibid. **31,** 284–90.

KERN, M., and NATALE, R. (1958). A diphosphopyridine nucleotidase and its protein inhibitor from *Mycobacterium butyricum*. *J. Biol. Chem.* **231,** 41–51.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., and RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. Ibid. **193,** 265–75.

NASON, A., KAPLAN, N. O., and COLOWICK, S. P. (1951). Changes in enzymatic constitution in zinc-deficient *Neurospora*. Ibid. **188,** 397–406.

—— —— and OLDEWURTEL, H. A. (1953). Further studies of nutritional conditions affecting enzymatic constitution in *Neurospora*. Ibid. **201,** 435–44.

—— OLDEWURTEL, H. A., and PROPST, L. M. (1952). Role of micronutrient elements in the metabolism of higher plants I. Changes in oxidising enzyme constitution of tomato leaves deficient in micronutrient elements. *Archives of Biochem. and Biophys.* **38,** 1–13.

NICHOLAS, D. J. D. (1957). The function of trace metals in the nitrogen metabolism of plants. *Ann. Bot.*, N.S. **21,** 587–98.

—— and GOODMAN, T. (1958). The effects of deficiencies of zinc and iron on some enzymes in *Neurospora*. *J. Exp. Bot.* **9,** 97–108.

—— and NASON, A. (1955). Role of molybdenum as a constituent of nitrate reductase from soya bean leaves. *Plant Physiol.* **30,** 135–8.

REED, H. S. (1946). Effects of zinc deficiency on phosphate metabolism of the tomato plant. *Amer. J. Bot.* **33,** 778–84.

—— (1947). A physiological study of boron deficiency in plants. *Hilgardia*, **17,** 377–412.

RICHARDS, F. J. (1938). Physiological studies in plant nutrition VIII. The relation of respiration rate to the carbohydrate and nitrogen metabolism of the barley leaf as determined by phosphorus and potassium supply. *Ann. Bot.*, N.S. **2,** 491–534.

SPENCER, D. (1954). The effect of molybdate on the activity of tomato acid phosphatases. *Austral. J. Biol. Sci.* **7,** 151–60.

TORRIANI, A. M. (1959). Effect of inorganic phosphate on formation of phosphatase by *E. coli*. *Fed. Proc.* **18,** 1344.

WALLACE, T. (1951). *Mineral deficiencies in plants*, 2nd edn., H.M. Stationery Office, London.

The Effect of Coumarin on Uptake of Water by Seeds

K. BLAIM

Institute of Soil Science and Cultivation, Pulawy, Poland

Received 1 June 1959

SUMMARY

There is a marked effect of coumarin on the water uptake of wheat seeds: uptake by the embryo is inhibited, but uptake by the endosperm is unaffected. Even in the early 'swelling' stage, water uptake by embryo and endosperm thus appears to involve two distinct mechanisms. It is suggested that the inhibitory effect of coumarin on germination is primarily due to its effect on water uptake by the embryo.

INTRODUCTION

THE interest in coumarin is due partly to its apparently universal occurrence in the plant world and partly to its physiological properties. Its 'blastocholine' (germination-inhibiting) properties were first demonstrated by Shreiner, Reed, and Skimov (1907), and have been studied more precisely by Velstra and Havinga (1943) with particular reference to *Lepidium sativum*; its selective phytocidal properties have been studied by Audus and Quastel (1947). A stimulative effect on germination has also been reported (Thimann and Bonner, 1949). In recent years lettuce and wheat seeds have also frequently been used as test material; wheat seeds have been found to be especially useful owing to their uniformity, which facilitates interpretation of results (Evenari, 1949).

The majority of studies on the influence of coumarin on seed germination have hitherto been largely confined to reporting the overall effects in given concentrations. Investigations on the mechanism of these effects have been fewer, and mainly concerned with the effect on enzyme systems (Poljakoff-Mayber, 1952, 1953*a* and *b*); the effect on water uptake does not appear to have been studied. However, the effect of growth regulators on permeability of protoplasm to water has been the subject of numerous studies; Pohl (1939), for example, observed that growth-promoting substances increase the permeability. Coumarin might therefore be expected to show the reverse effect. That this is the case is strongly suggested by the experiments of Moewus (1949) on the effect of coumarin on the bending of oat coleoptiles; and Guttenberg *et al.* (1952, 1954), using *Rhoeo discolor* and potato tuber parenchyma, have demonstrated specifically that coumarin decreases permeability to water. It therefore seemed reasonable to investigate whether the inhibiting effect of coumarin on germination is mediated by a decreased inflow of water to the cells.

MATERIALS AND METHODS

The material consisted of wheat seeds supplied by the Cereal Breeding Division of the Institute of Plant Breeding and Acclimatization, Puławy, and obtained from the 1957 harvest. All investigations were carried out on the winter varieties 'Brzostowianka', 'Kujawianka', 'Puławska Winter', and 'Puławska 684'; the spring varieties 'Ostka Chłopicka' and 'Ostka Puławska'; and a Puławka wheat, obtained from an 1895 harvest, which had completely lost its germination capacity. Germination capacity for the remaining varieties was about 95 per cent. Measurements were made on 6 replicates of 100 seeds.

Water uptake was determined by soaking the weighed seed samples for 24 hours in water or in a 10^{-3} M. solution of coumarin in water. Seeds were then removed from the plates, placed on dry filter-paper to remove excess moisture, and reweighed. The significance of the mean difference between the uptakes of water and coumarin for each variety was determined by a *t* test.

RESULTS

The results are presented in Table I. It will be seen that the effect of coumarin is statistically significant but small, and the question arises as to whether this relatively small difference can constitute the direct cause of inhibition of germination. The last two entries in the table show that old seeds which have lost their capacity for germination, and normal seeds which have been deprived of their embryos, show no effect of coumarin; and it therefore seems highly probable that the influence of coumarin on water intake of seeds relates only to the embryo. The precise separation of the embryo from the endosperm in wheat is technically very difficult, and a method of successive approximation was therefore used; this consisted of analysing embryos with differing amounts of endosperm. The results are shown in Fig. 1, from which it will be clear that the smaller the amount of endosperm left with the embryo, the greater the influence of coumarin on water uptake. At a 100-seed weight of about 100 mg. (i.e. with a very small amount of endosperm) the water uptake of control samples exceeds 300 per cent., whereas in the presence of coumarin the uptake reaches only 200 per cent., a fall of 33 per cent. in total uptake. As the 100-seed weight in-

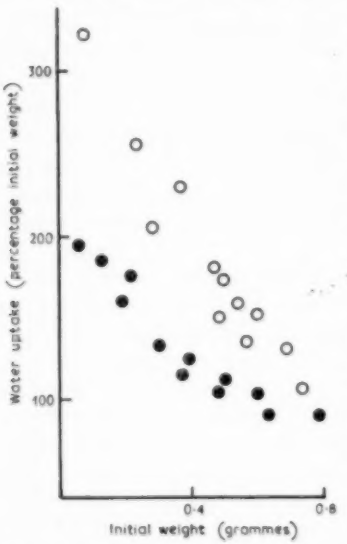


FIG. 1. Abscissa represents weight of 100 embryos with varying amounts of endosperm. Open circles, water control; closed circles, 10^{-3} M. coumarin.

creases this quantity itself falls, so that in the case of the whole grain, as can be seen from Table I, it does not even reach 6 per cent.

TABLE I

Effect of 10^{-3} M. coumarin on water uptake by wheat seed (uptake in 24 hours as percentage increase of initial weight of 100 seeds; 6 replicates of each treatment)

Variety	Per cent. water uptake: water (m_1)	Per cent. water uptake: coumarin (m_2)	m_1-m_2	t
(i) *Normal winter*				
Brzostowianka	46·08	43·44	2·65	11·0
Kujawianka	45·23	41·29	3·94	5·9
Puławska Winter	48·46	45·41	3·05	7·2
Puławska 684	44·85	42·63	2·22	6·9
(ii) *Normal spring*				
Ostka Chłopicka	48·47	45·89	2·58	4·9
Ostka Puławska	50·15	44·46	5·71	7·7
(iii) *Inviable (1895)*				
Puławska	40·65	40·84	−0·19	—
(iv) *Embryos removed*				
Brzostowianka	45·92	45·81	0·11	—

DISCUSSION

This investigation has demonstrated (*a*) that there is an effect of coumarin on water uptake by seeds, and (*b*) that there is a difference in the character of this uptake as between embryo and endosperm even in the early stage of swelling. The evidence for this difference is the fact that coumarin inhibits only water uptake by the embryo, and has no effect on uptake by the endosperm. We may therefore doubt the validity of the two stages of seed water uptake distinguished by Atkins (1909), viz. stage I—water uptake connected with swelling of colloids, and stage II—water uptake connected with germination and due to osmosis. It seems that the distinction between these two stages is more closely linked with considerations of space than of time. It is more probable that already during the stage of swelling water uptake by the embryo is connected with the growth and development of cells, and hence has a distinctly physiological character, whereas uptake by the endosperm is connected exclusively with imbibition by colloids. The investigation here reported thus confirms the opinions and results of Pohl and Guttenberg (loc. cit.), according to which one of the means by which growth regulators react upon the plant organism is the induction of changes in the permeability of the protoplasm. It further suggests that the immediate cause of the inhibitive effect of coumarin should be sought for in its influence on the permeability of the protoplasm of the cells of the embryo.

LITERATURE CITED

ATKINS, W. R. G. (1909). The absorption of water by seeds. *Sci. Proc. Roy. Dublin Soc.*, N.S. **12**, 35.

AUDUS, L. J., and QUASTEL, L. H. (1947). Coumarin as a selective phytocidal agent. *Nature*, **159**, 320.

EVENARI, M. (1949). Germination Inhibitors. *Bot. Rev.* **15**, 153.

GUTTENBERG, H., and BEYTHIEN, A. (1952). Über den Einfluss von Wirkstoffen auf die Wasserpermeabilität des Protoplasmas. *Planta*, **40**, 46.

—— und MEINL, G. (1954). Über die Veränderung der Wasserpermeabilität von Kartoffelknollen. *Planta*, **43**, 571.

MOEWUS, F. (1949). Die Wirkung von Wuchs- und Hemmstoffen auf die Kressewurzel. *Biol. Zbl.* **68**, 58.

POHL, R. (1939). Über die primäre Wirkung des Wuchsstoffes bei der Zellstreckung. *Ber. Dtsch. Bot. Ges.* **57**, 22.

POLJAKOFF-MAYBER, A. (1952). Germination inhibitors and plant enzyme systems. *Bull. Res. Council Israel*, **2**, 239.

—— (1953*a*). *Palest J. Bot.* **6**, 60.

—— (1953*b*). Ibid. **6**, 101.

SCHREINER, O., REED, H. S., and SKIMOV, K. (1907). *U.S. Dept. Agric. Bur. Soils. Bull.* **47**.

THIMANN, K. V., and BONNER, W. D. Inhibition of plant growth by protoanemonin and coumarin and its prevention by BAL. *Proc. Nat. Acad. Sci.* **35**, 272.

VELSTRA, H., and HAVINGA, E. (1943). On the physiological activity of unsaturated lactones. *Enzymologia*, **11**, 373.

Electron Microscopic Observations of the Structure of Sieve-connexions in the Phloem of Angiosperms and Gymnosperms

C. E. L. HEPTON[1] AND R. D. PRESTON

Botany Department, University of Leeds

Received 1 February 1960

SUMMARY

The sieve elements of *Pinus silvestris* L., *Sorbus aucuparia* L., *Vitis vinifera* L., and *Cucurbita pepo* L. have been examined electron microscopically in ultra-thin section, and the structures of the corresponding sieve areas or sieve plates have been described and compared. In *Pinus* the sieve areas contain groups of connecting strands which enter the wall from the lumen side as individuals but coalesce within it in the *median cavity*. This cavity has developed by wall breakdown in the middle lamella and primary wall region and corresponds to the *median nodule* visible under a light microscope. Neither in this nor in the other species observed is there any visible closing membrane.

Structural differences between the four species are shown to suggest that the major evolutionary trend in the evolution of specialized conducting strands has been the enlargement of the connecting strands from groups of small separate strands to a smaller number of larger strands as the median cavity becomes enlarged to form a canal through the wall.

The connecting strands appear invariably to be dense, highly osmiophilic and continuous with the cytoplasmic surface of the cell. No signs of micropores or of other tubular structure in the strands have been found. The structures thus revealed are more nearly compatible with active transport of materials across the sieve plate than they are with any purely physical mechanism. It is suggested that they are incompatible with any mass flow hypothesis.

INTRODUCTION

THE mechanism by which solutes move through the phloem of higher plants is one of those perennial problems of plant physiology which have developed an extensive literature without leading to a final solution. The sieve-tube elements discovered by Hartig in 1837 have generally been considered as the main conduits for transport and the few opponents who have expressed contrary views have done so largely because of the lack of a coherent theory of movement. It is doubtful if any workers in this field now challenge the actual path of transport and this is perhaps the sole point of agreement.

Each of the various theories of transport reflects clearly the views held by its particular advocates on the detailed structure of the sieve elements. One of the earliest and most disputed problems here has been the nature of the 'connecting strands', i.e. the material traversing the wall between two adjacent

[1] Present address: Cornwall Technical College, Redruth, Cornwall.

sieve elements (Esau, 1950). Münch (1943) has illustrated the four distinct concepts. The oldest is that due to Hill (1908), who regarded the connecting strands as tubular structures, composed of peripheral cytoplasm with a central vacuole continuous across the sieve plate from one cell vacuole to the next. This structure has figured largely in the 'Mass flow theory' of Münch. Crafts, who is responsible for much of our knowledge of sieve elements, has never accepted this view but has held that the connecting strands consist of 'solid cytoplasm' (Crafts, 1939) and claimed to have demonstrated that the tonoplast disappears as the sieve element matures. Finally, Mangenot and later Salmon have expressed the view that there is no cytoplasmic continuity between the sieve elements, each half of the connecting strands being invested by its own individual plasma membrane (see Esau, 1948*a*). A somewhat similar view is expressed by Volz (1952) for the plasmodesmata and the connexions in gymnosperms and some angiosperms.

A decision between these different interpretations is clearly desirable. This is particularly so following the work of Currier *et al.* (1955) on the permeability relations of the cytoplasm in sieve elements. Crafts has for long strongly sustained the thesis that mature sieve elements cannot be plasmolysed and he has used this to support his argument that the sieve elements are freely permeable at maturity. Several other workers in this field have claimed that mature sieve elements can be plasmolysed if special precautions are taken (Curtis and Asai, 1939; Rouchal, 1941), but Crafts (1951) has not fully accepted this evidence. The extensive reinvestigation of this problem by Currier *et al.* (1955) seems to leave no doubt that mature sieve elements can be plasmolysed under suitable conditions. This conclusion has removed a powerful argument for the Mass Flow theory unless evidence can be brought forward for ready permeability in the longitudinal direction. The major resistance to flow will be at the sieve plates and the present paper is concerned with the detailed structure of the connecting strands in the sieve-plate region using the technique of ultra-thin sectioning and electron microscopy. It describes the results of an investigation made during 1956 (Hepton, 1957); since that time electron microscopical findings have been published from other laboratories, notably that of Schumacher (1959). These will be discussed with the present results.

MATERIALS AND METHODS

Observations have been made on one gymnosperm (*Pinus sylvestris* L.) and three angiosperms (*Sorbus aucuparia* L., *Vitis vinifera* L., and *Cucurbita pepo* L.). This material was chosen partly on the grounds of suitability for the special techniques to be used and partly in relation to certain sieve-tube problems. Thus the four species were chosen on account of the absence of fibres and excessive amounts of oxalate crystals from the phloem, for ease of sectioning. *Cucurbita* was an obvious choice on the grounds of the presence of large pores in a simple transverse sieve plate. *Sorbus* represents the other extreme of the angiosperm type with many small pores in a compound sieve plate on a steeply sloping end wall and is of additional interest in view of

Volz's (1952) findings that the sieve-element structure of this species is close to the gymnosperm type. *Vitis* occupies a somewhat intermediate position and is of special value in showing the phenomenon of reactivation. Finally, *Pinus* was chosen as a representative of the somewhat different condition in the *Abietineae* group of the gymnosperms.

In general the methods were as outlined below though slight modifications were used with each material as indicated in the appropriate section. Pieces of stem about 6 in. long were immersed in a pretreatment solution consisting of 0·2–0·3 M. sucrose buffered to pH 7 with KH_2PO_4 and K_2HPO_4 and containing 0·001 per cent. $Ca(NO_3)_2$ as recommended by Currier *et al.* (1955). The function of this solution is to lower the turgor pressure within the sieve elements. After about 2 hours small strips of bark were removed at the cambium and, from the inner surface of these, thin sections were cut with a razor kept moist with the pretreatment solution. The sections were transferred to a 1 per cent. osmic acid solution buffered to pH 7·3 with veronal acetate following the method of Palade (1952). The sections were subsequently dehydrated in alcohol and embedded in methacrylate in the now familiar manner. From the polymerized blocks sections were cut, using either a Hodge-type microtome or a modified Cambridge rocker. The sections were in some cases treated to remove the methacrylate and in others examined directly in the electron microscope.

NOMENCLATURE

In view of the confusion that exists in the literature it seems desirable to say something about the nomenclature to be used when referring to wall perforations in sieve elements.

The walls of young growing cells often show a beaded appearance in optical section, a feature characteristically seen in cambial strips (Priestley *et al.* 1933). The thin places in such walls are usually referred to as *primary pit fields*. Within these primary pit fields plasmodesmata are grouped together. Gardiner (1897) distinguished such 'pit threads' from the 'wall threads' where such regular grouping does not occur. In the specialized conducting cells of the phloem the groups of plasmodesmata are, at some stage, associated with a substance called callose. About the time the callose appears certain important changes occur in the protoplasm. The nucleus breaks down and the slime body or bodies, commonly present as discrete structures, become dispersed. At this stage the plasmodesmata become much more conspicuous, often thicker, and show a greater affinity for stains. The conspicuous plasmodesmata now associated with callose are referred to by Esau (1950) as *connecting strands*. The primary pit fields, following these modifications, are now referred to as *sieve areas* (Cheadle and Whitford, 1941). Where the connecting strands of the sieve areas are particularly well developed that part of the cell wall is spoken of as a *sieve plate*. If the sieve plate bears uniformly-distributed pores it is a *simple sieve plate*, but where there are regions of wall between the discrete sieve areas it is called a *compound sieve plate*. Sieve areas differ in the size of

their connecting strands. Where these are very small the name *sieve field* has been applied. Since transitional stages occur, Esau (1948*a* and *b*) recommends the use of the single term *sieve area*; the less specialized ones being referred to as *type B sieve areas*, the more specialized ones found in sieve plates as *type A sieve areas*. We shall show, however, that there is probably an important structural difference between the two types.

Finally, the sieve cells of *Pinus* differ from the angiospermous sieve elements studied in that they possess true secondary walls (Abbe and Crafts, 1939), and the areas lie in depressions and may be designated pits; the name *sieve pit* was in fact given to them by Abbe and Crafts. The sieve pits have a compound structure analogous to a compound sieve plate, that is they consist of groups of small sieve areas. Since these appear to correspond structurally to the type B sieve areas in angiosperms this is an additional reason for not using the term 'sieve plate' in gymnosperms as is done in the older literature (Hill, 1901).

RESULTS

Pinus sylvestris L.

The sieve cells of *Pinus* are long and fusiform, bearing a large number of sieve pits distributed over the radial walls. The detailed structure of these is difficult to make out owing to their complexity and small size. It is a great tribute to the earlier workers that they were able to give a fairly clear picture of the structure.

Our present knowledge is largely due to the work of Hill (1901), who also reviewed the earlier work. According to him, each sieve pit in surface view has a circular to oval outline surrounding a number of small polygonal or rounded sieve areas. A transverse section of the sieve pit shows that each sieve area corresponds to a 'cork' of callose perforated by a number of holes through which the cytoplasm passes. The two 'corks', one from each adjacent cell, are separated at the middle lamella by a structure known as a median nodule. Apart from the fact that this shows up as a darkly staining dot in transverse section nothing is known about its structure, though Hill interpreted it as a swelling of the middle lamella. He was unable to say whether it allowed for continuity of cytoplasm across the wall from one sieve cell to the other or represented a small imperforate membrane.

Volz (1952) examined the sieve cell connexions in *Larix* by electron microscopy of macerated fragments and concluded that the sieve connexions were closed by a thin membrane. This conclusion was not, however, based on sections and her Fig. 1, showing a sieve pit of *Larix*, is open to a different interpretation in terms of the present results.

This very brief description of the sieve pits of *Pinus*, as interpreted by earlier workers, makes it clear that there are several points about which further information and clarification is desirable. These are:

(*a*) The structure of the median nodules so often figured in descriptions of gymnosperm sieve cells.

(*b*) The continuity or discontinuity of cytoplasm across the sieve pit membrane.

The material used for the present investigation was taken from a fully grown tree of *Pinus sylvestris*. Sections were cut from the inner surface of strips of bark, separated at the cambium, taken from the main trunk. These were fixed directly in 1 per cent. osmic acid in the field, transferred to the laboratory for embedding, and sectioned in the tangential longitudinal direction. The methacrylate was removed from the sections by passage through amyl alcohol and amyl acetate and the sections were then shadowed with Pd/Au.

Pl. I, Fig. 1 represents the appearance of a sieve pit seen in section, and the nature of the median nodules at once becomes apparent. They are clearly distinct cavities within the two primary walls plus middle lamella forming the pit membrane and within each of them there is material resembling the peripheral cytoplasm. The sharp outline of the cavities makes it certain that they represent a localized breakdown of the middle lamella and part of the primary wall of the two adjacent sieve cells and are not due to sectioning. The central of the three cavities shows several 'branching' strands. The nature of these is not obvious from this figure alone, but a study of Pl. I, Fig. 2 makes their interpretation more certain. This is an electron micrograph from a thick section and it shows both that the protoplasm is continuous across the wall and that the strands entering the wall pass into the median cavity. The material within the cavities has reacted with osmium just as has the rest of the cytoplasm. This is interpreted to mean that here a number of connecting strands fuse together. There is some evidence that the nodule cavities arise by a gradual breakdown of wall material in the middle lamella region. It is of interest that such a breakdown was described by Gardiner (1897) in the endosperm cells of *Tamus communis* and later referred to by him when describing the median nodules of *Pinus* (Gardiner and Hill, 1901). The inner surface of the wall of the sieve cells away from the sieve pit has a lining of amorphous material. The precise nature of this is not clear but it bears a strong resemblance to the callose deposits seen in later sections.

Pl. I, Fig. 1 also shows that there is no closing membrane such as Volz (1952) has described for *Larix*. If such a membrane were present it would traverse the cavities since these include the middle lamella region. The most likely explanation of Volz's results is that her maceration technique separated the cells at the middle lamella. In that case a face view of the sieve pit would reveal depressions corresponding to the halves of the median cavities just as is shown in her Fig. 1 for Larch.

The conclusions reached from tangential longitudinal sections were confirmed by radial longitudinal sections which are not illustrated here.

From the evidence presented it would appear that the structure of the sieve pits of *Pinus* may be briefly summarized as follows. Each consists of a number of groups of connecting strands, the sieve areas, the strands of any one group entering the wall from the cell lumen individually but coalescing within the median cavity. Each group is associated with callose at the wall surface but

the exact relationship here is not yet clear. It is further evident that the sieve pit in the region of the sieve area is not closed by a membrane, so that the cytoplasm is continuous across the wall from one sieve cell to the next.

Sorbus aucuparia L.

In the study of this material, intact rooted cuttings were immersed in the pretreatment solution after the removal of the outer corky layer. Further treatment was as described under Material and Methods.

Under a light microscope, numerous sieve areas may be seen on the radial walls of the sieve elements. These have been compared with those of gymnosperms by Hüber (1939) and, more recently, Volz (1952) has reported that the individual pores through which the connecting strands pass are closed by a membrane in the middle lamella region as she also claimed for *Larix*. The sieve elements of *Sorbus* were thus regarded as being closer to the gymnosperm type than to the angiosperm types. This similarity was said to be further shown by the absence of a true end wall in the sieve elements of both gymnosperms and *Sorbus*.

It has not proved possible to agree with either of these points. Pl. II, Fig. 3 shows a sieve area in tangential longitudinal section of the phloem and leaves no doubt that the connecting strands are continuous across the intervening wall. Further, there is no evidence of the median cavities found in *Pinus*; in fact the connecting strands are in every way similar to those in the sieve areas of the other angiosperms examined. As regards the second point, the individual sieve elements have a distinct, if steeply sloping, end wall and the sieve areas are largely confined to this region. The radial longitudinal walls bear only a few much smaller sieve areas. The sloping end walls of *Sorbus* should therefore be described as compound sieve plates and the sieve elements referred to as sieve-tube elements as in other angiosperms, and not as sieve cells (*Siebzellen*) as is done by Volz (1952).

Vitis vinifera L.

Vitis is one of a few woody plants the sieve-tube elements of which show activity over more than one season; in *Vitis* itself they may be reactivated in as many as four successive seasons (Esau, 1948*b*). In most plants at the end of the season in which the sieve tubes are formed the sieve plates finally become covered by a thick layer of callose (definitive callus) and the cell contents disappear. In *Vitis*, callose is formed within the pores and a thick layer also extends over the sieve plate, but this largely disappears the following spring and the sieve-tube elements resume activity. It is because of this behaviour that Esau (1948*b*) refers to such callose as *dormancy* callose. While the phenomenon of reactivation of sieve elements is uncommon, it has been described for a number of plants (Esau, 1950) and must be taken into account when considering translocation as a whole. It seemed, therefore, worthwhile to examine the sieve plates of *Vitis* during the period of reactivation. The end walls of the individual sieve elements are generally sloping in *Vitis* but, since

the initials often undergo transverse divisions before differentiation, some also have transverse end walls (Esau, 1948*b*). The sieve plates on the sloping end walls are of the compound type with the plate facing radially; those on transverse end walls are simple.

Rooted cuttings were used and strips of phloem taken as soon as the cambium resumed activity as judged by its readiness to separate from the xylem (Priestley *et al.*, 1933). The phloem strips were then treated as described under Methods.

Pl. III, Figs. 4–7 shows four serial sections of a compound sieve plate in section. For ease of reference the parts of these have been numbered 1 to 4 in the serial sections. They are seen in Pl. III, Fig. 4 to be heavily callosed and no trace of connecting strands is visible. Pl. III, Fig. 5 shows parts of two connecting strands at position 1 and from Pl. III, Fig. 6 it is clear that these completely traverse the sieve plate. They are extremely narrow since they have almost vanished in Pl. III, Fig. 7. As judged by section thickness these particular strands cannot exceed about 1,000 Å. (0·1 μ) in diameter. This extreme tenuity probably accounts for the report by some previous workers of their absence during the dormant period (Esau, 1948*b*).

Pl. III, Fig. 7 shows connecting strands in positions 2 and 3, the one at position 2 being ruptured at the left-hand side. Attention is drawn to the similar appearance of the connecting strands and the outermost layer of cytoplasm. It will be particularly noticed that the dense material of the connecting strand at position 2 is limited to the pore and does not spread out into the cytoplasm of the elements.

Pl. IV, Figs. 8 and 9 show sections through a simple sieve plate in an element having a transverse end wall. Here again the sieve plate is heavily callosed and it is interesting that neither here nor in the previous series of sections does the callose of the plate show the layering mentioned by Esau (1948*b*). As regards reactivation, the increased size of the connecting strands is probably significant (despite the fact that this is a simple plate on a transverse wall), especially when coupled with other changes. A point of interest is the changed appearance of the callose of the plate at the points where it is penetrated by the connecting strands. This is seen very clearly in Pl. IV, Fig. 9, where the depressions in the callose suggest a digestive action by the enlarging strands on the callose of the sieve plate.

Here again the similarity can be seen between the surface layer of the cytoplasm and the connecting strands. The thickness of the connecting strands within the pores makes it clear, however, that they are more than just continuations of the outer surface of the cytoplasm, though even where they are relatively thick they appear homogeneous and cannot be resolved into tubular structures, with an outer layer of cytoplasm around central slime, as pictured by Hill (1908). It seems that in *Vitis*, during the period of reactivation, the connecting strands consist predominantly of a material having a strong affinity for osmium. This in fact appears to be a characteristic reaction of connecting strands in general and will be referred to again later.

Pl. V, Figs. 10–12 show serial tangential longitudinal sections through a longitudinal wall of a sieve-tube element; the areas are not on a plate and correspond to Esau's type B. The sieve areas show very clearly the callose cylinders surrounding single, or small groups, of deeply stained connecting strands. In Pl. V, Fig. 10 the unit marked with an arrow is particularly interesting; three small callose cylinders each with its central connecting strand are enclosed in a cavity which differs by its dark outline from surrounding callose cylinders. If this same group is examined in the next serial section, Fig. 11, only one callose cylinder can be seen but it encloses several connecting strands. These observations suggest that in type B sieve areas of angiosperms median cavities develop in the wall much in the same way as has been demonstrated for *Pinus*. This finding accords with the observations of Hill (1908) that median nodules occur both in the sieve pits of *Pinus* and in the less well-developed sieve areas of angiosperms (i.e. Esau's type B). Apparently in the present case the callose cylinders at the lumen side of the wall 'branch' as they penetrate the wall towards the middle lamella region, now broken down to form the median cavity. As regards the strands, this 'compound' structure for type B sieve areas would make them correspond essentially to the observations described for *Pinus*. Hill (1901) thought that the callose cylinders of *Pinus* branch as they approach the middle lamella. In the present study such 'branching' of callose cylinders was not seen in *Pinus* but appears to occur in type B areas of *Vitis*.

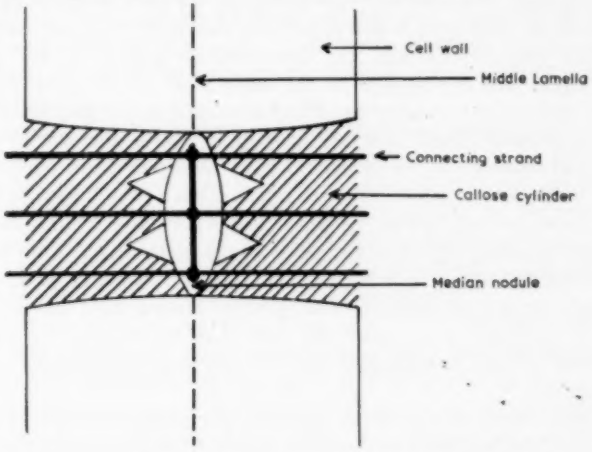
The 'branching' is probably only apparent; the true situation is more likely to be due to a greater tendency for callose cylinders to coalesce near the lumen side than towards the middle lamella, so that connecting strands within the median cavity, with (*Vitis*) or without (*Pinus*) distinct callose cylinders, are represented next the cell lumen by a single callose cylinder around one or few connecting strands. This may be shown diagrammatically as in Text-fig. 1. Almost all the sieve areas examined in *Vitis* show single callose cylinders containing more than one connecting strand. The callose cylinders are not continuous across the wall but show a discontinuity in the middle lamella region, a result which confirms Hill's (1908) earlier observation. The connecting strands, however, are continuous across the wall and no membrane closing the pores can be detected.

Cucurbita pepo L.

This has been one of the classical materials for the study of sieve plates. Hill (1908) used it to study sieve-plate development, and Crafts (1932) made a detailed study of the ontogeny of the sieve-tube elements in this plant. It seemed therefore appropriate to re-examine the connecting strands using the present technique.

The initial results obtained with this material were published by us in a previous communication (Hepton *et al.*, 1955) and only a few additional points will be referred to here. At that time no reference was made to the

obvious difference in density between the pore contents and the general cytoplasm. This was thought to be due largely to the contraction that had occurred in the pore material. In the light of our later experience this is certainly not the full explanation. The pore material is always denser than the rest of the



TEXT-FIG. 1. Diagrammatic representation of the structure of a pore in a sieve area of *Vitis* as seen in section. (Drawn about 50,000 ×.)

cell cytoplasm and this appears to be due to the presence of some osmiophilic substance within the pores. The large size of the sieve-tube elements in *Cucurbita* made them favourable objects for study with the light microscope. Now that it is possible to examine material with the electron microscope size is of less importance. It has, in fact, the great disadvantage that disturbance of the contents seems more likely to occur. This is well seen in Pl. VI, Fig 13, where obvious displacement of the contents has taken place despite the pretreatment. This probably occurred in the initial cutting of the material and led to the use of rooted cuttings in the later work. Pl. VI, Fig. 13 shows a striking resemblance to a section shown by Crafts (1939, Fig. 2). Crafts interpreted the appearance as a tearing away of the connecting strand from its position at the sieve plate and there seems no doubt that this is the correct interpretation. The membrane visible cannot be that suggested by Salmon (see Esau, 1948*a*) since there is no sign of it in the pores at the edges of the plate.

DISCUSSION

The results of the present investigation have a bearing on both anatomical and physiological problems related to translocation and these will be discussed separately.

The principal anatomical problem at issue is the nature of the connecting strands between the sieve elements of angiosperms and sieve cells of

gymnosperms. None of the sections examined in the present investigation provide any evidence to support the view that the protoplasts of the individual sieve elements are surrounded by individual plasma membranes in such a way as to interrupt continuity of cytoplasm across the sieve plate. Continuity has on the contrary been demonstrated repeatedly for the protoplasts of all the sieve areas examined, both type A and type B, as well as for the plasmodesmata of parenchyma cells, Pl. VI, Fig. 14. This lends no support to the conclusion of Volz (1952) that the strands in the sieve areas of gymnosperms and of *Sorbus*, as well as the parenchyma cells of *Dracaena*, are closed by a fine membrane, a conclusion based on a face view of the pores and not on the much more critical sectional views presented here. The picture of plasmodesmata as 'tentacle-like extensions of the protoplast' (Esau *et al.*, 1957) penetrating the wall is also not supported. All previous workers have treated the material with various swelling agents to demonstrate plasmodesmata. They are shown here in material which has been fixed without swelling and appear as undoubted continuous strands. The present results have further brought forward no evidence to support the view that the connecting strands are protoplasmic tubules as suggested by Hill (1908) and repeatedly referred to in the literature.

The observations do, however, support the views of Crafts and others (Crafts, 1939) that the pores of the sieve plate are filled with cytoplasm and, in particular, are of precisely the kind published by Schumacher (1959). They nevertheless raise strong doubts as to whether the connecting strands can be considered as consisting of ordinary cell cytoplasm and strictly merit the name plasmodesmata. We would support the terminology of Esau (1950) and retain the term 'connecting strand' as there is good evidence on the grounds of response to stains that they contain some additional material which has a strong affinity for osmium.

It has long been recognized in the literature that the connecting strands show increased chromaticity at the time the slime bodies disintegrate, and there have been attempts to associate this slime with the connecting strands. Thus Hill (1908) gives a drawing showing the vacuolar slime passing through the protoplasmic tubules of the sieve plate. Esau (1948*b*) also suggested that slime is a normal constituent of the connecting strands in *Vitis*, and thought this might be an indication of vacuolar continuity. We have found no evidence of any vacuolar slime; the dense contents, both in *Vitis* and other material examined, is confined to the pores and shows no connexion with any vacuolar material. This is well seen in Pl. III, Fig. 7.

Some evidence as to the nature of the connecting strands can be obtained from the stains which have been used to demonstrate them. Meeuse (1941) in his review of plasmodesmata refers to Tangl's original method using iodine-potassium iodide solutions. Tangl found the plasmodesmata to stain in the same way as did the surface membrane, and consequently believed that plasmodesmata are merely continuations of the plasma membrane. The present results support this. Iodine seems to have been the basis of most later staining

methods. Thus it was used by Crafts (1931) and to some extent by Mühldorf (1937). It appears from the latter's work, as well as from that of Livingston (1935), that iodine reacts with the stains used to give a precipitate. It was for this reason that Mühldorf thought it gave unreliable results. Mühldorf found that the connecting strands could be partly removed by treatment with proteolytic enzymes. They then stained black with osmium. From this he concluded that they consist of protein as well as some lipids. Recently Scott (1955), using Sudan Black, reported that both plasmodesmata and the cytoplasmic surface give a fat reaction.

These results thus lead to the conclusion that the connecting strands consist of both protein and fat components. Now slime is said to be proteinaceous and only Salmon is reported to hold the view that it is in part lipoidal (Esau, 1948*a*). The dense black reaction with osmic acid given by the pore material strongly suggests the presence here of unsaturated fats, a point not explicable in terms of slime as generally understood. The possible meaning of this staining reaction will be referred to later in the discussion.

It might be mentioned here that Esau *et al.* (1957) expressed doubt as to whether present methods allow of a distinction being made between slime and cytoplasm. This statement hardly seems valid in view of the obvious distinction seen here particularly in the unshadowed sections. In the previously published photographs (Hepton *et al.*, 1955) the material was shadowed with Pd/Au but in the ones presented here, particularly *Vitis*, a clear distinction occurs between the cytoplasm and the pore material. If the pores had a tubular sheath of cytoplasm this ought to be visible. As little contraction has occurred it seems reasonable to conclude that such a sheath is absent. The resemblance between the pore material and the outer layer of cytoplasm is, however, striking and it is tempting to suggest that they are of a similar nature.

The evidence presented suggests that the major trend in the evolution of the specialized conducting cells, as far as it concerns the connecting strands, has been in the enlargement of these from groups of separate strands to a smaller number of large strands. The way in which this may have occurred is as follows. In ordinary parenchyma cells such as phloem parenchyma, the two protoplasts of adjacent cells are united only by their surface layers; this can be seen in Pl. VI, Fig. 14. Attention is particularly drawn to the small cavities in the primary wall. In the sieve cells of *Pinus* we have many groups of plasmodesmata and it has already been shown that individual strands of any one group open into a common cavity, the median cavity. This is a much larger and more distinct structure than that in the wall between parenchyma cells. It is now suggested that these median cavities enlarged to become canals through the wall as found in the type A sieve areas of the angiosperms. If this is correct the connecting strands have been derived by fusion of several small ones. Thus the pores will now contain a number of fused membranes. If these membranes have a high lipoid content this could account for their dense appearance.

A detailed review on the physiology of phloem has recently been published

(Esau *et al.*, 1957) so that it is necessary here to make only a brief statement of the bearing of the present observations on the main theories of transport. The four principal theories assume movement respectively by:

1. Surface diffusion
2. Cytoplasmic streaming
3. Mass or pressure flow
4. Protoplasmic or activated diffusion.

The idea of rapid transport by surface diffusion was developed by van den Honert (1932) and is still regarded as a possible mechanism (van Overbeek, 1956). It is, however, difficult to reconcile with the structure of the system. The original objection of the lack of suitable liquid/liquid interfaces (see Faraday Soc. Sym. 1948) is probably no longer a major obstacle to its acceptance. Electron microscopy of thin sections has revealed the presence of numerous surfaces within the cell cytoplasm (Sjöstrand, 1953), and the fine reticulations seen in the plates of *Vitis* presented here possibly represent such surfaces. The main resistance to flow, however, must occur within the pores of the sieve areas and it is precisely here that no evidence can be found for their existence.

The major objection to streaming as a mechanism of transport remains the inability of most workers to demonstrate that it occurs in mature sieve elements (Currier *et al.*, 1955). Nevertheless, the well-known sensitivity of these cells to the necessary manipulations cannot entirely rule out the possibility that streaming could well play some part in the overall process. This mechanism offers no explanation of rapid movement along pores now known to be completely filled.

The mass-flow hypothesis does not seem to receive any support from the present results. In the first place, it is clear that there is no vacuolar continuity across sieve plates or areas and this in itself eliminates the mass-flow hypothesis in its original form. Evasion of this difficulty by assuming a mass movement of the whole contents (Münch, 1943) is equally impossible. If the pore material has a special constitution, as our results seem to demonstrate, then this part must remain fixed in position and any movement must be through this material. Crafts (1951) clearly accepts the immobility of the cell contents, since according to him they are denatured and the solutes move through a permeable cytoplasm. With the demonstration by Currier *et al.* (1955) that the mature sieve elements can be plasmolysed, this assumption by Crafts is, however, not substantiated. To overcome this difficulty the suggestion was made by Currier *et al.* that the connecting strands might have a structure which would make them readily permeable and would allow easy flow in a longitudinal direction. The present results, far from indicating a permeable nature of the connecting strands, suggest a contrary view. Certainly no micropores such as Crafts (1951) postulated have been found.

The movement of solutes as the result of an active process would suggest the presence of an active agency within the sieve tubes themselves. Experiments

supporting such a view have come recently from the work of Kursanov (1952, 1956). The major difficulty with this theory has been the absence of evidence as to the site of the active process. The present work indicates a likely site, namely the densely staining connecting strands. In all cases investigated, these appear as distinct, darkly stained, homogeneous regions confined to the pores of the sieve areas. The greatest resistance to solute movement will occur where the cross-sectional area is reduced, i.e. at the pores, so it is here that an active process will be of most value. This would make it reasonable that, as shown by Esau and Cheadle (1955), the sieve-element mother cells often undergo transverse divisions and hence increase the number of sieve plates in a given length of stem. Now the electron microscopical appearance of the connecting strands is very similar to that of the outermost layer of the cytoplasm in osmium fixed material. Darkening in osmium fixatives is largely confined to unsaturated fats, to some amino-acids with double bonds (e.g. histidine and tryptophane) and other unsaturated compounds. Compounds of this type are commonly associated with oxidation-reduction centres such as chloroplasts and mitochondria. It is therefore possible that the connecting strands contain systems capable of energy transfer just as the cytoplasmic surfaces are said to do.[1] Movement through the pores might thus be active transport of the kind known to be operative for the movement of materials into cells. A possible mechanism along these lines has already been discussed by Spanner (1958). It remains to account for movement along sieve-tube lumina from one set of connecting strands to the next. It is still possible that streaming could play an important part here in bringing about mixing, and the evolutionary trend in the shortening of the sieve elements would render this easier.

ACKNOWLEDGEMENT

The authors wish to express their gratitude to Miss L. I. Scott, late of the Botany Department, University of Leeds, for advice and discussion during the preparation of this paper.

LITERATURE CITED

Abbe, L. B., and Crafts, A. S. (1939). Phloem of white pine and other coniferous species. *Bot. Gaz.* **100**, 695.

Cheadle, V. I., and Whitford, N. B. (1941). Observations on the phloem in the Moncotyledoneae. I. The occurrence and phylogenetic specialization in the structure of the sieve tubes in the metaphloem. *Amer. J. Bot.* **28**, 623.

Crafts, A. S. (1931). Technic for demonstrating plasmodemata. *Stain Technol.* **6**, 127.

—— (1932). Phloem anatomy, exudation and transport of organic nutrients in cucurbits. *Plant Physiol.* **7**, 183.

—— (1939). The relation between structure and function of the phloem. *Amer. J. Bot.* **26**, 172.

—— (1951). Movement of assimilates, viruses, growth regulators, and chemical indicators in plants. *Bot. Rev.* **17**, 203.

Currier, H. B., Esau, K., and Cheadle, V. I. (1955). Plasmolytic studies of phloem. *Amer. J. Bot.* **42**, 68.

[1] This was pointed out to us by Professor W. T. Williams of Southampton in a private communication.

CURTIS, O. F., and ASAI, G. N. (1939). Evidence relative to the supposed permeability of sieve tube protoplasm. *Amer. J. Bot.* **26,** 16 supplement.
ESAU, K. (1948*a*). Some anatomical aspects of plant virus disease problems. *Bot. Rev.* **14,** 413.
—— (1948*b*). Phloem structure in the grapevine, and its seasonal changes. *Hilgardia,* **18,** 217.
—— (1950). Development and structure of the phloem tissue II. *Bot. Rev.* **16,** 67.
—— and CHEADLE, V. I. (1955). The significance of cell divisions in differentiating secondary phloem. *Acta Bot. Neerl.* **4,** 348.
—— CURRIER, H. B., and CHEADLE, V. I. (1957). Physiology of phloem. *Ann. Rev. Plant. Physiol.* **8,** 349.
GARDINER, W. (1897). The histology of the cell wall with special reference to the mode of connection of cells. *Proc. Roy. Soc.* **62,** 106.
—— and HILL, A. W. (1901). The distribution and character of connecting threads in the tissues of *Pinus sylvestris* and other allied species. *Phil. Trans. Roy. Soc.* B. **194,** 83.
HEPTON, C. E. L. (1957). *The structure of phloem in relation to its function.* Ph.D. Thesis, Leeds.
—— PRESTON, R. D., and RIPLEY, G. W. (1955). Electron microscopic observations on the structure of the sieve plates in Cucurbita. *Nature,* **176,** 868.
HILL, A. W. (1901). The histology of the sieve tubes of *Pinus*. *Ann. Bot.* **15,** 575.
—— (1908). The histology of the sieve tubes of angiosperms. Ibid. **22,** 245.
HONERT, T. H. VAN DEN (1932). On the mechanism of the transport of organic materials in plants. *Proc. Kon. Akad. Wet. Amsterdam,* **35,** 1104.
HÜBER, B. (1939). Das Siebröhrensystem unserer Bäume und seine jahreszeitlichen Veränderungen. *Jahrb. Wiss. Bot.* **88,** 176.
KURSANOV, A. L. (1952). *Botan. Zhur. S.S.R.* **37,** 585.
—— (1956). Recent advances in plant physiology in the U.S.S.R. *Ann. Rev. Plant. Physiol.* **7,** 401.
LIVINGSTON, L. G. (1935). The nature and distribution of plasmodesmata in the tobacco plant. *Amer. J. Bot.* **22,** 75.
MEEUSE, A. D. J. (1941). Plasmodesmata. *Bot. Rev.* **7,** 249.
MÜHLDORF, A. (1937). Das plasmatische Wesen der pflanzlichen Zellbrücken. *Bot. Centrbl. Beih.* **56,** 171.
MÜNCH, E. (1943). Durchlässigkeit der Siebröhren für Druckströmungen. *Flora,* **36,** 223.
OVERBEEK, J. VAN (1956). Absorption and translocation of plant regulators. *Ann. Rev. Plant Physiol.* **7,** 355.
PALADE, G. E. (1952). A study of fixation in electron microscopy. *J. Exp. Med.* **95,** 285.
PRIESTLEY, J. H., SCOTT, L. I., and MALINS, M. E. (1933). A new method of studying cambial activity. *Proc. Leeds Phil. Soc.* **2,** 365.
ROUCHAL, E. (1941). Untersuchungen über die Protoplasmatik und Funktion der Siebröhren. *Flora,* **35,** 135.
SCHUMACHER, W. (1959). Zur Anatomie des Siebröhrenplasmas bei *Passiflora caerulea*. *Ber. deut. Bot. Ges.* **72,** 176–9.
SCOTT, F. M. (1955). The distribution and physical appearance of fats in living cells. Introductory survey. *Amer. J. Bot.* **42,** 475.
SJÖSTRAND, F. S. (1953). Electron microscopy of mitochondria and cytoplasmic double membranes. *Nature,* **171,** 30.
SPANNER, D. C. (1958). The translocation of sugars in sieve tubes. *J. Exp. Bot.* **9,** 332–42.
VOLZ, G. (1952). Elektronenmikroskopische Untersuchungen über die Porengrössen pflanzlicher Zellwände. *Mikroskopie,* **7,** 251.

HEPTON *and* PRESTON—PLATE 1

FIG. 1. Electron micrograph of a tangential longitudinal section (T.L.S.) through the phloem of *Pinus sylvestris*. Shadowed Pd/Au. Magn. 6,000 ×. Sieve pit in section showing median cavities and branched connecting strands

FIG. 2. As in Fig. 1 above, methacrylate not removed, unshadowed. Magn. 4,600 ×. Note continuity of connecting strands

HEPTON *and* PRESTON—PLATE II

FIG. 3. Electron micrograph of T.L.S. through the phloem of *Sorbus aucuparia*. Methacrylate not removed, unshadowed. Magn. 17,600 ×. Sieve area showing continuous connecting strands

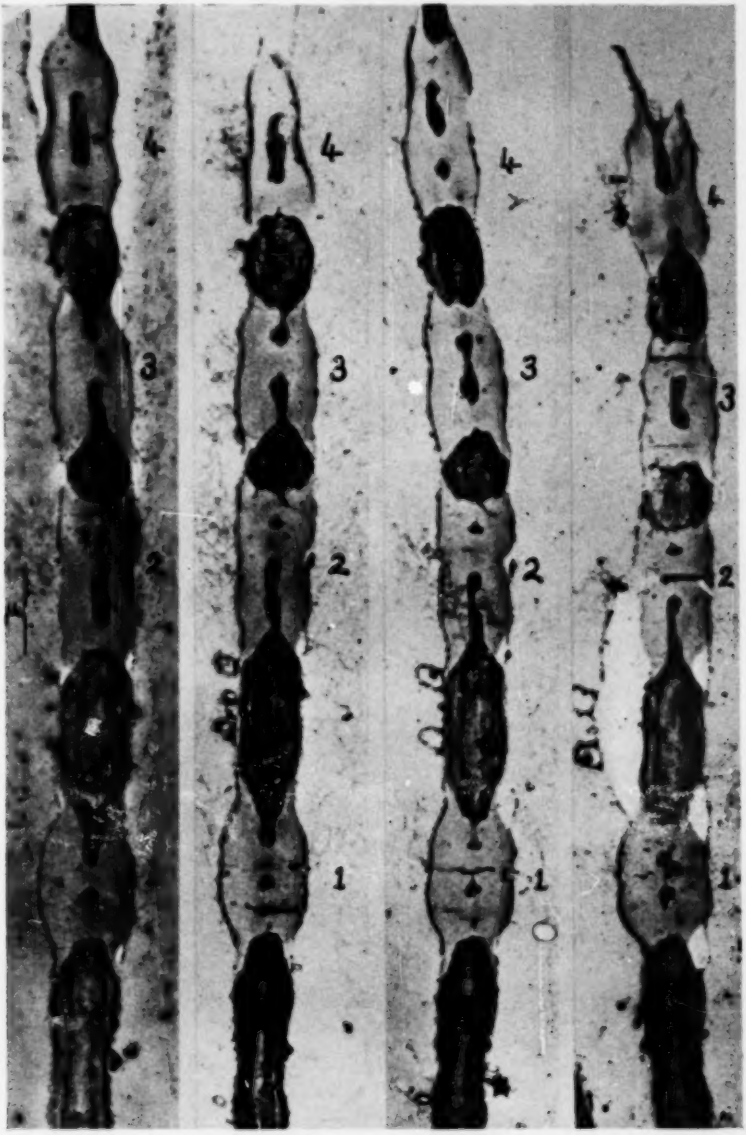
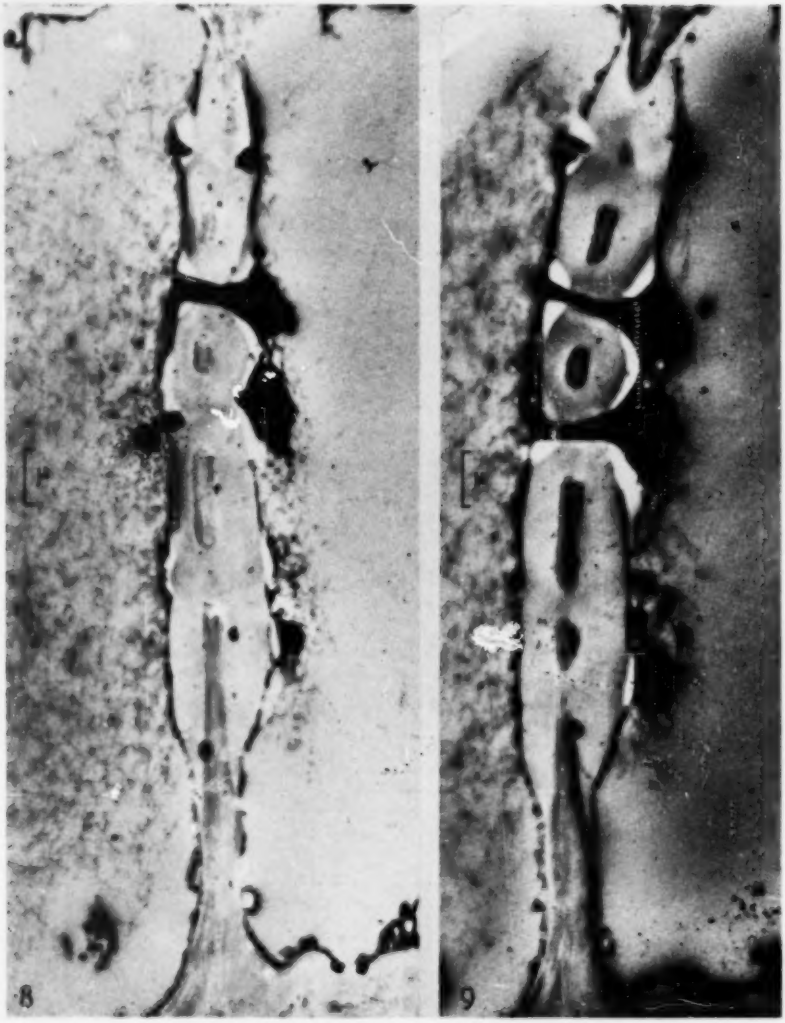


FIG. 4 FIG. 5 FIG. 6 FIG. 7

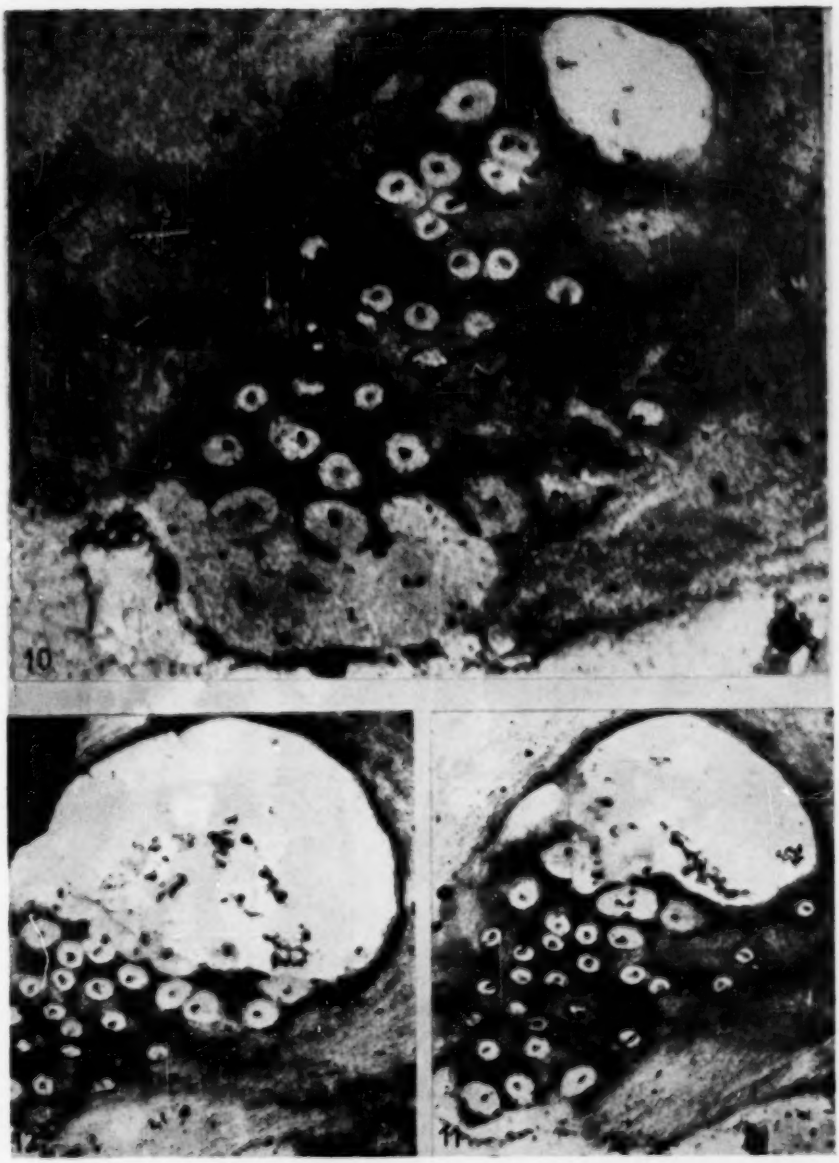
HEPTON *and* PRESTON—PLATE III

FIGS. 4–7. Electron micrographs of 4 serial T.L.S.'s through the phloem of *Vitis vinifera*. Methacrylate not removed, unshadowed. Magn. 4,750 ×. Compound sieve plate showing callosed pores and fine connecting strands



HEPTON *and* PRESTON—PLATE IV

FIGS. 8 and 9. Electron micrographs of T.L.S.'s through the phloem of *Vitis vinifera* showing sieve plates in section. Methacrylate not removed, unshadowed. Magn. 7,500×. Note the prominent connecting strand, apparently continuous with the plasma membrane, and the depressions in the callose where the strands enter

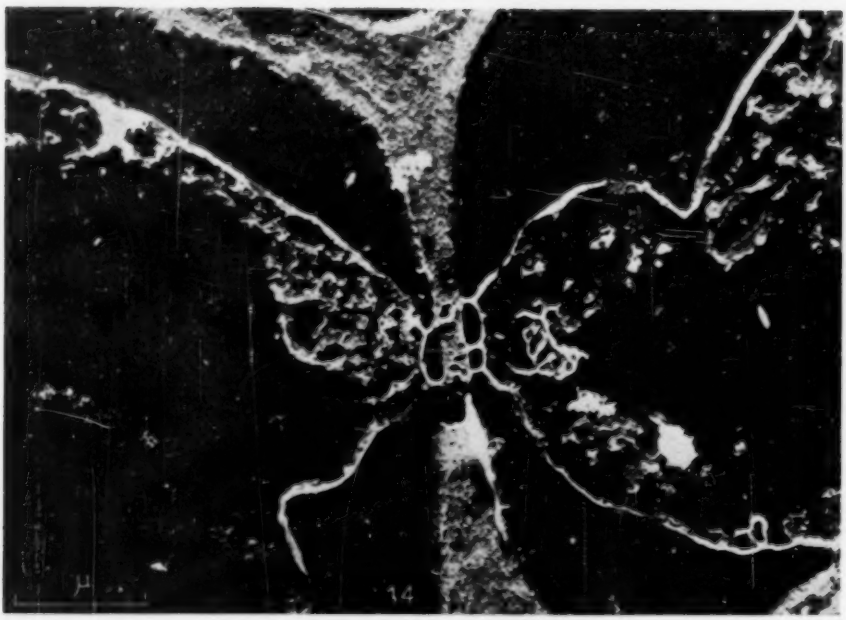


HEPTON *and* PRESTON—PLATE V

FIGS. 10–12. Serial sections showing a sieve area *of Vitis vinifera* in surface view. Methacrylate not removed, unshadowed

FIG. 10. Magn. 22,500×. The arrow indicates several callose cylinders entering a nodule cavity

FIGS. 11 and 12. Magn. 10,500×. The arrow marks the same pore marked in Fig. 10



HEPTON *and* PRESTON—PLATE VI

FIG. 13. Electron micrograph of longitudinal section through the phloem of *Cucurbita pepo*. Methacrylate removed, shadowed Pd/Au. Magn. 4,500×. Sieve plate in section. Note the displacement of the cytoplasm through the central pore

FIG. 14. Electron micrograph of section through the phloem of *Cucurbita pepo*. Methacrylate removed, shadowed Pd/Au. Magn. 19,500×. Plasmodesmata between two phloem parenchyma cells

Studies in Morphogenesis of *Brassica oleracea* L.

II. THE COMPARATIVE MORPHOLOGY OF TYPICAL HEADING-TYPE CABBAGE PLANTS AND NON-HEADING ROGUES

C. NORTH

Scottish Horticultural Research Institute, Mylnefield, Invergowrie, Dundee

Received 22 March 1960

SUMMARY

Non-heading rogue plants of January King cabbage have as many leaves, and as short vegetative axes, as typical heading plants. Leaves of rogues are generally narrower than those of a corresponding age from headed plants sampled in the same crop, and their dry matter content is higher. Rogue plants of January King sampled in the winter had more advanced flower development than headed plants. The significance of these differences for the explanation of the phenomenon of heading is discussed.

INTRODUCTION

In an earlier paper (North, 1957) some morphological features of five varieties of cabbage that mature at different rates were examined to find which growth characteristics are associated with the phenomenon of heading —i.e. the development of a compacted mass of folded leaves. In this present paper the association of certain morphological features with this phenomenon is examined further by comparing headed plants with non-headed plants taken from the same crop.

MATERIALS AND METHODS

Plants for analysis. In Britain, non-headed plants occur in 'mature' crops of many strains of cabbage, especially those of the varieties January King and Christmas Drumhead. Such plants develop flower stems early in the second year of growth and thus complete their life cycle without ever forming a head. Seed obtained by self-fertilizing non-headed plants of January King at Mylnefield has given considerably higher proportions of non-headed plants than were present in the original stocks. By continuing selection for this character over several generations, Detjen and McCue (1933) isolated stocks from the variety Volga which bred true for non-heading. Thus it seems that non-heading is genetic and that plants which exhibit this character in mature crops must be looked upon as rogues.

However, this rogue trait may be modified by environmental conditions. Sneddon[1] has found that ramets taken from non-headed plants in mature

[1] Personal communication from J. L. Sneddon, The National Institute of Agricultural Botany, Cambridge.

crops, and maintained in a vegetative condition throughout the winter by keeping them in a temperature above 60° F., will sometimes produce heads the following year. Nevertheless, the range of environmental conditions to which market crops of January King and Christmas Drumhead are usually subjected has no appreciable modifying effect on the rogue types: the frequency with which they occur in different localities and seasons does not vary greatly.

Plants for analysis were sampled during 1956 and 1957, in the neighbourhood of Edinburgh and Dundee, from four market-garden crops of the variety January King, and one experimental crop of Christmas Drumhead. Seed was sown in April, and the plants were grown under normal market-garden conditions. The crop of Christmas Drumhead consisted of two stocks selected by the National Vegetable Research Station, Wellesbourne: one of these was known to segregate about 80 per cent. rogues, and the other a high proportion of heading plants. Rows of plants of each of the two strains were grown in a randomized-block layout.

Except for one sample of young plants of Christmas Drumhead, all plants were taken from crops which had ceased vegetative growth in autumn and winter.

Morphological features observed. The configuration of leaves around the shoot apices was observed in 20 young plants, selected at random from each of the stocks of Christmas Drumhead, by dissecting the growing points under a stereo-microscope. Shoot apices of mature plants of January King were also examined in a similar manner; most of these had entered the reproductive stage, and the state of the shoot apex in relation to flower formation, in both rogue and headed types, was scored according to the following scale:

1. No sign of flower initiation.
2. Shoot apex broad, with axillary buds showing.
3. Flower initials just visible with stereo-microscope.
4. Very small flower buds visible with stereo-microscope but not with naked eye.
5. Very small flower buds just visible with naked eye.
6. Small flower buds easily visible with naked eye.
7. Flowering shoots ½ to 8 in. long.
8. Flowering shoots greater than 8 in. long.
9. Some flower buds showing yellow.
10. Several flowers open.

(Not all these categories were used, but they are included here for further reference.)

Rogue and headed plants selected at random from mature crops were also compared for fresh weight, number of leaves (dead and living), length of main axis (measured from soil level to shoot apex). The length of successive internodes was measured in both types of plants taken from one crop of January King. The fresh weight, dry weight, length, and greatest width of successive leaves was recorded for rogues, and for headed plants, of January King.

EXPERIMENTAL RESULTS

All comparisons in January King were made between plants taken from mature crops, since in the early stages of growth it was not possible to distinguish between rogue genotypes and potential heading plants. The two

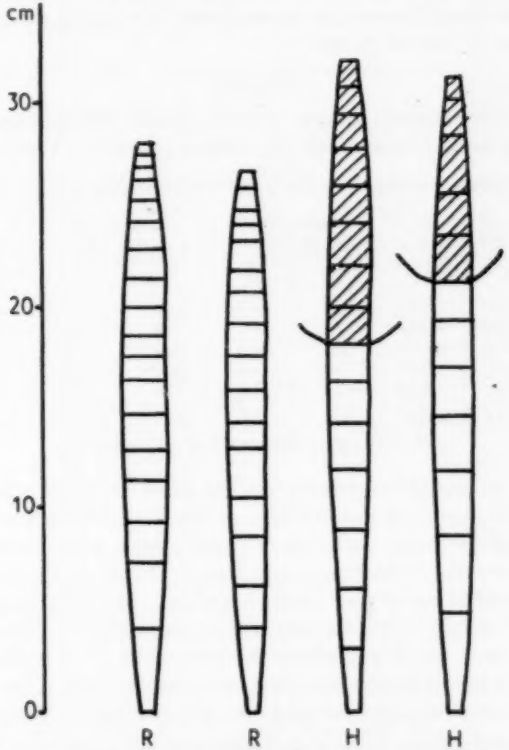


FIG. 1. Diagram showing internodes of main axes of rogue (R) and headed (H) plants taken from the same mature crop of January King cabbage. Shaded areas represent parts of the stem bearing folded leaves; horizontal lines are drawn at five-node intervals.

strains of Christmas Drumhead, however, provided an opportunity for comparing these types at an early stage of development, because the progenies were either mainly rogues, or mainly headed plants. An examination of the shoot apices of 20 plants of each of the two strains, when they had about 10 unfolded leaves (i.e. before any showed outward signs of head formation), gave no evidence of a difference in the configuration of the embryonic leaves; in all plants, the leaves were folded over the growing point in an apical bud (cf. Stage 2 of stem apex, North (1957)). Some 80 per cent. of plants of the 'rogue' strain would have developed into non-headed individuals, so that it

may be concluded that rogues, as well as potential heading plants, develop an apical bud.

Table I shows two clear differences between rogue and headed plants: (1) the fresh weight of headed plants was, in each crop, significantly greater than that of rogue plants, and (2) although nearly all the plants sampled in January had initiated flowers, flower development was slightly more advanced in rogues than in headed plants.

TABLE I

Some morphological features of headed plants (H) compared with non-headed rogue plants (R). (Mean values for 10 plants)

Cabbage January King (JK) and Christmas Drumhead (CD)

Crop	Fresh wt. per plant (g.)		Number of leaves formed		Length of main axis		State of growing point	
	R	H	R	H	R	H	R	H
JK 1	1,180 •	1,502	111	110	230	217	4·1 •	2·6
JK 2	849 •	1,369	81	87	195	224	4·7 •	2·9
JK 3	1,364 •	2,468	90	93	241	295	6·0	5·3
JK 4	1,501 •	1,827	99	91	275	293	6·2	5·9
CD	1,019 •	1,224	111 •	89	120	117	—	—
Mean	1,373 •	2,032	101	96	251	277	5·2 •	4·2

• Difference significant at 5% point.

There was no consistent difference in the number of leaves formed by the two types of January King and although, on the whole, the main axis of headed plants was slightly longer than that of rogue plants, the differences between the mean measurements were never significant. Therefore, the average spacing of leaves of both types of plant must have been about the same within any given crop. However, this information does not indicate whether the distribution of the stem nodes was similar for both types. This feature was examined and it was found that in December the first 60 leaves to be formed were usually more widely spaced in headed than in rogue plants. Two stems of each type from January King are shown diagramatically in Fig. 1; they had been sampled at random, but show a rather larger difference of stem length than is usual between the types.

In Christmas Drumhead the mean number of leaves of rogue plants was significantly higher than that of headed plants. This is inconsistent with the findings for January King, but too much importance should not be attached to this fact since the two types of Christmas Drumhead represent different stocks which had been selected for the heading character and may at the same time have been unintentionally selected for differences in the rate of leaf initiation.

Dry weight, percentage dry weight of fresh weight, width, and length : width ratio of successive leaves of rogues and headed plants are shown in Fig. 2 for the crops JK 3 and JK 4. The leaves of headed plants contain a relatively higher proportion of water than those of rogue plants, and this may largely

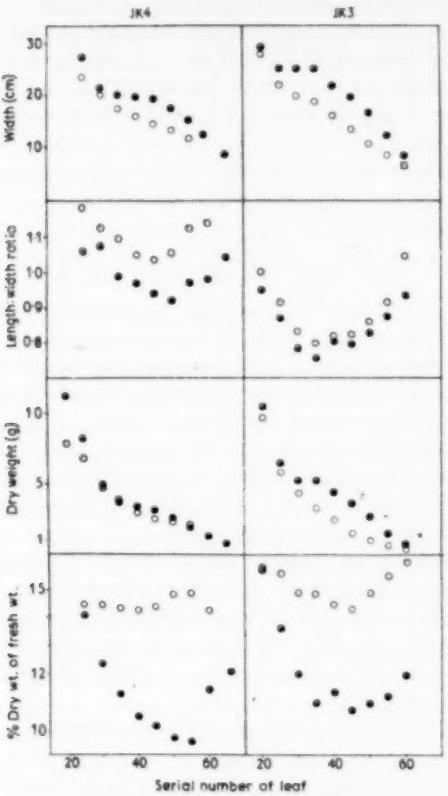


FIG. 2. A comparison between certain leaf features of rogue and headed plants taken from two crops (JK 3, JK 4) of January King cabbage. Each point represents a mean for ten plants. ● = folded leaves of headed plants, ⊖ = unfolded leaves of headed plants, ○ = leaves of rogue plants. The serial number of the leaf refers to the order in which the leaves were formed; that of the oldest leaf is 1.

account for the significantly higher fresh weight recorded for headed plants. The dry weights of leaves of both rogue and headed plants were similar in crop JK 4, although there were considerable differences in the fresh weights of the two types of this crop. Since the leaves constitute 70–90 per cent. of the total dry weight of a cabbage plant, one may assume that the differences in fresh weight observed in crop JK 4 were due largely, if not entirely, to differences in water content of the leaves.

Fig. 2 also shows that the leaves of rogues had a higher length: width ratio than those of a similar serial number (and, since the total number of leaves

may be concluded that rogues, as well as potential heading plants, develop an apical bud.

Table I shows two clear differences between rogue and headed plants: (1) the fresh weight of headed plants was, in each crop, significantly greater than that of rogue plants, and (2) although nearly all the plants sampled in January had initiated flowers, flower development was slightly more advanced in rogues than in headed plants.

TABLE I

Some morphological features of headed plants (H) compared with non-headed rogue plants (R). (Mean values for 10 plants)

Cabbage January King (JK) and Christmas Drumhead (CD)

Crop	Fresh wt. per plant (g.)		Number of leaves formed		Length of main axis		State of growing point	
	R	H	R	H	R	H	R	H
JK 1	1,180 *	1,502	111	110	230	217	4·1 *	2·6
JK 2	849 *	1,369	81	87	195	224	4·7 *	2·9
JK 3	1,564 *	2,468	90	93	241	295	6·0	5·3
JK 4	1,501 *	1,827	99	91	275	293	6·2	5·9
CD	1,019 *	1,224	111 *	89	120	117	—	—
Mean	1,373 *	2,032	101	96	251	277	5·2 *	4·2

* Difference significant at 5% point.

There was no consistent difference in the number of leaves formed by the two types of January King and although, on the whole, the main axis of headed plants was slightly longer than that of rogue plants, the differences between the mean measurements were never significant. Therefore, the average spacing of leaves of both types of plant must have been about the same within any given crop. However, this information does not indicate whether the distribution of the stem nodes was similar for both types. This feature was examined and it was found that in December the first 60 leaves to be formed were usually more widely spaced in headed than in rogue plants. Two stems of each type from January King are shown diagramatically in Fig. 1; they had been sampled at random, but show a rather larger difference of stem length than is usual between the types.

In Christmas Drumhead the mean number of leaves of rogue plants was significantly higher than that of headed plants. This is inconsistent with the findings for January King, but too much importance should not be attached to this fact since the two types of Christmas Drumhead represent different stocks which had been selected for the heading character and may at the same time have been unintentionally selected for differences in the rate of leaf initiation.

Dry weight, percentage dry weight of fresh weight, width, and length: width ratio of successive leaves of rogues and headed plants are shown in Fig. 2 for the crops JK 3 and JK 4. The leaves of headed plants contain a relatively higher proportion of water than those of rogue plants, and this may largely

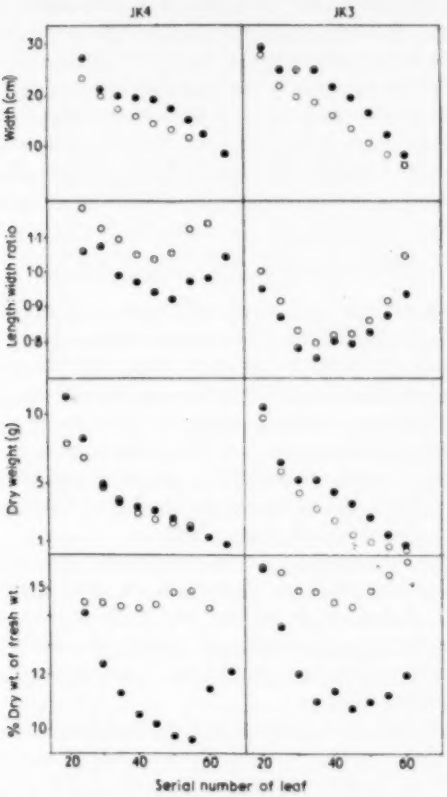


FIG. 2. A comparison between certain leaf features of rogue and headed plants taken from two crops (JK 3, JK 4) of January King cabbage. Each point represents a mean for ten plants. ● = foided leaves of headed plants, ◑ = unfolded leaves of headed plants, ○ = leaves of rogue plants. The serial number of the leaf refers to the order in which the leaves were formed; that of the oldest leaf is 1.

account for the significantly higher fresh weight recorded for headed plants. The dry weights of leaves of both rogue and headed plants were similar in crop JK 4, although there were considerable differences in the fresh weights of the two types of this crop. Since the leaves constitute 70–90 per cent. of the total dry weight of a cabbage plant, one may assume that the differences in fresh weight observed in crop JK 4 were due largely, if not entirely, to differences in water content of the leaves.

Fig. 2 also shows that the leaves of rogues had a higher length:width ratio than those of a similar serial number (and, since the total number of leaves

formed is about the same for both types, of similar age) from headed plants in the same crop. The differences shown here are not quite significant, but measurements on three other crops of the same variety previously examined for this characteristic also clearly demonstrated such a difference. Leaves of headed plants were also broader in actual width than corresponding ones from rogue plants. The difference was most pronounced between those leaves within the head and similarly aged leaves of rogue plants, but unfolded leaves of headed plants were also broader than those of a corresponding age from rogue plants.

DISCUSSION AND CONCLUSIONS

The head of a cabbage is a mass of compacted leaves which can only develop when a large number of leaves are produced on a comparatively short stem. A head could not form, for example, on a kale plant (*Brassica oleracea acephala*) because the leaves are too widely spaced. Observations recorded in this paper show that non-heading rogues of January King and Christmas Drumhead cabbages have as many, and as closely spaced, leaves as headed plants: in fact there is some evidence that leaves of rogues are more crowded on the stem than those of typical plants. One must therefore look for some other reason why rogues do not form heads.

Outer leaves of a cabbage head are folded; that is, their margins are clasped around their younger neighbours. They form a kind of skin, within which the inner leaves continue to grow in volume until the head is compact and mature. The leaves of a rogue of a comparable age to the outer head leaves of a typical plant are not folded, and it is in this respect that rogues differ essentially in structure from headed plants.

Detjen and McCue (1933) recognized two different processes of head formation in cabbage which they described as 'direct' and 'indirect'. In the direct type, the apical bud—which is composed of folded leaves and structurally resembles a very small cabbage head—continues to increase in size and leaf number until it becomes a full-sized head. In the indirect type the head was thought to be formed by the refolding of some leaves which have already unfolded. In spite of careful observation, no evidence of the indirect type of heading could be observed by the author in the varieties January King and Christmas Drumhead and it seems that head formation in these varieties is always direct.

Apical buds, reminiscent of small cabbage heads, were formed by all the young plants of rogue strains as well as in the potential heading plants which were examined. Thus rogues must differ from headed plants in that, under conditions usually provided for the crop, all the leaves of the apical bud eventually unfold, whereas in heading plants some of them do not.

The continued unfolding of successive leaves of rogues is associated with certain leaf characteristics. Leaves of rogues are usually narrower than those of a similar age from headed plants, both in actual width and also in width in relation to length, and they have a lower water content. The differences in

actual width and in water content are greatest between leaves in the head and those of a corresponding age from rogue plants, but they also exist, to a lesser degree, between the unfolded leaves of headed plants and their counterparts in rogues. This suggests that the differences are not solely an effect of leaf unfolding, such as a change in microclimate brought about by the shelter of adjacent leaves, but that they may be associated with some factor or factors which control leaf unfolding. Differences between the two types of plant in the shape of leaf as measured by the length:width ratio seem to be more or less independent of leaf position and there are therefore stronger grounds for believing that this feature is associated with the factor which causes leaf unfolding.

The relationship between leaf shape and heading in cabbage is of special interest. A similar relationship has been observed in lettuce (Bensink, 1959) and studied in this crop as a phenotypic expression; a low length:width leaf ratio being associated with head formation. Now there is some evidence that rogues may be considered as very late-maturing genotypes, for ramets of rogues will sometimes form heads if the period of vegetative growth is prolonged by inhibiting flower initiation. Thus it might be expected that varieties which mature late generally have narrower leaves than those which mature early, and there is evidence that this is true (North, 1960).

The observed difference in moisture content of leaves of rogues and headed plants is of a similar order to that found between the folded and unfolded leaves of a headed plant. In typical plants, high moisture content of head leaves is associated with a high sugar content. Although the chemical composition of the leaves of rogues was not determined, it seems probable that they may contain less sugar than those of headed plants. This would be consistent with the findings of Ito (1936), who associated the onset of heading in lettuce with an increase in soluble carbohydrate content of the plant, provided the nitrogen supply was adequate. Rogue plants of cabbage could be those which are relatively inefficient carbon assimilators, and an accumulation of sugars in young leaves of cabbage may in some way be associated with those factors which determine their shape and prevent them from unfolding.

Rogues differed from headed plants not only in the shape and configuration of leaves, but also in their more advanced flower development. There is some slight evidence that non-heading may be associated with a tendency to early flower initiation, for the variety January King, which is especially prone to form rogues, also seems to initiate flowers earlier than some other varieties (North, 1957). Observational evidence strongly suggests that the same is also true of Christmas Drumhead, for this variety tends to form premature bolters when grown in the north of Britain. Thus the rogues in these two varieties might be premature seeders similar to the non-heading 'rosette seeders' described by Miller (1929). The evidence to support this view is not, however, very strong. Plants of the 'rogue' strain of Christmas Drumhead did not flower earlier than those of the heading strain which had been selected from the same source. It seems rather that the small differences in flower development

observed in January King may be an effect of, rather than a feature associated with the cause of, heading. It is possible that the developing head serves to insulate the growing point from extremes of temperature so that it is subjected to shorter accumulated periods below the threshold temperature for flower initiation than one which has no protection from a head.

ACKNOWLEDGEMENTS

I wish to acknowledge the assistance of Messrs. L. H. Frith and H. Taylor in recording some of the information presented in this paper. Dr. A. G. Johnson provided the seed of the two strains of Christmas Drumhead cabbage and Mr. David Lowe, C.B.E., and Mr. G. Swarbrick permitted the sampling of their crops of January King cabbage. Dr. M. R. Sampford, of the A.R.C. Unit of Statistics at Aberdeen, kindly advised on the statistical examination of some of the data.

LITERATURE CITED

BENSINK, J. (1959). Heading of lettuce (*Lactuca sativa* L.) as a morphogenetic effect of leaf growth. *Rept. 15th Int. hort. Congr.* (In press.)

DETJEN, L. R., and MCCUE, C. A. (1933). Cabbage characters and their heredity. *Bull. agric. Exp. Sta. Univ. Delaware*, No. 180.

ITO, H. (1936). The development and the chemical changes of lettuce as influenced by temperature, light, and nutrient supply, with special reference to head formation. *J. Hort. Ass. Japan*, **7**, 58.

MILLER, J. C. (1929). A study of some factors affecting seed stalk development in cabbage. *Bull. Cornell Agric. Exp. Sta.* **488**.

NORTH, C. (1957). Studies in morphogenesis of *Brassica oleracea* L. I. Growth and development of cabbage during the vegetative phase. *J. Exp. Bot.* **8**, 304.

—— (1960). Relationship between leaf shape and head formation in cabbage (*Brassica oleracea* L. var. capitata). *Rept. 15th Int. Hort. Congr.* (In press.)

The Nature of the Resistance of Oats to the Take-all Fungus

III. DISTRIBUTION OF THE INHIBITOR IN OAT SEEDLINGS

ELIZABETH M. C. TURNER

St. Anne's College, Oxford

Received 18 December 1959

SUMMARY

The distribution in oat seedlings of an inhibitor of fungal growth and respiration is described. The inhibitors from roots and leaves differ in that that from roots has a bright blue fluorescence under ultra-violet light, while in other respects they behave similarly; it is suggested that they differ in structure of a part of the molecule not concerned in the biological activity. The inhibitor is present in high concentration in the meristems of roots and leaves, one root tip extracted in 2 ml. water being sufficient to reduce growth of *O. graminis* to 50 per cent. of the control; yield from other parts of the seedling is much less. A quick assay technique based on growth-rate of mycelium is described, also a method for direct identification of inhibitory zones on chromatograms which avoids the necessity for elution.

INTRODUCTION

AN inhibitor of fungal growth and respiration present in roots and leaves of oat seedlings has been described (Turner, 1940, 1952, 1956), and it was suggested that it is concerned in resistance of oats to the take-all disease of wheat and barley caused by *Ophiobolus graminis*. The closely related *O. graminis* var. *avenae* is also sensitive to the inhibitor but it can infect oats. The inhibitor is thermostable, inactivated by reduction by glutathione or ascorbic acid, more slowly inactivated by oxidation, soluble in 80 per cent. methanol and ethanol, slightly soluble in dry methanol and ethanol and in water, and insoluble in ether.

Some further information on distribution of the inhibitor in oat seedlings and on the reaction to it of the two varieties of *O. graminis* and of *Neurospora crassa* is given below.

MATERIALS AND METHODS

The fungi used were the following:

Ophiobolus graminis (W and W_2) isolated from wheat in Oxfordshire in 1957,

O. graminis var. *avenae* (O_2, O_3, and O_5) from oats kindly supplied by Dr. Bradley-Jones from Glamorgan, Wales, and

Neurospora crassa, St. Lawrence strain, wild type, mating type A, kindly supplied by Dr. Fincham, The University, Leicester.

The culture medium for *O. graminis* was that described by White (1941) with the following composition per litre:

$MgSO_4$ 1 g. (2 g. hydrated), KH_2PO_4 1 g., Na_2HPO_4 3 g., $FeCl_3$ trace, glucose 20 g., NH_4NO_3 5 g., biotin 1 mg., thiamin 1 mg.

The glucose was added after autoclaving. *N. crassa* was grown in Fries minimal medium. Both media were used with or without 1·5 per cent. agar.

Inhibitor was obtained from oat seedlings grown (after soaking in water for 2 hours) on muslin supported on stainless steel ¼-in. grids over water in polythene dishes. For some experiments segments of known numbers of seedlings were used, but general supplies were obtained as follows:

(*a*) roots of seedlings 5–7 days old were cut, a dish providing about 12 g. fresh weight of roots, and ground in a mortar. They were extracted several times with water to give a total volume after filtration through glass wool of 250 ml. The extract was shaken with 10 g. powdered Whatman No. 1 filter-paper which had previously been washed with 80 per cent. methanol, acetone, and distilled water; most of the active material was adsorbed on to the paper, from which it was removed with 80 per cent. ethanol. The extract was assayed and concentrated by heating to a volume with an ED_{50} against *N. crassa* at a dilution of 1 in 50.

(*b*) The leaf extract could be prepared in a similar way except that, to remove the pigments, the paper was washed with chloroform before elution. However, this method gave a preparation from the tops with too many impurities and generally the tops (about 15 g. from a single dish) were extracted as above, the extract was boiled, the coagulum collected by centrifugation, washed with chloroform, and extracted with 80 per cent. methanol, most of the inhibitor being adsorbed on the particles of the coagulum. Columns of ion exchange resins such as Amberlite IR45 in the OH form removed inhibitor from solution satisfactorily, apparently by adsorption, elution being effected only by methanol or acetone at concentrations in water of 40 per cent. or more; these solvents, however, remove from the resin a blue fluorescent material also inhibitory to fungal growth, so the method was abandoned.

The following rapid method of assay was devised, using mycelial growth as a criterion for measuring inhibition rather than spore germination, which is less affected by this inhibitor: rectangles 2·5 × 0·5 cm. were marked on glass slides with a diamond; 0·3 ml. of the liquid to be assayed was added to an equal volume of double strength nutrient agar, the mixture was heated until liquid, taken up with a heated 1 ml. pipette, and 0·1 ml. was streaked on to the marked rectangles of each of 3 replicate slides; these agar strips were inoculated at the centre with a disk 3 mm. in diameter cut with a drawn-out piece of glass tubing from just behind the growing edge of a colony in a petri-dish, and the slides were stored in glass slide boxes lined with damp filter-paper, 9 slides

to a box. Growth was measured as diameter after 18 hours at room temperature for *N. crassa* and 36 hours for *O. graminis*. Because of the short growth-period few precautions for maintaining sterility were necessary.

Chromatograms of extracts were run in various solvents (ascending). Assay of eluates for inhibitory activity was difficult because of inactivation of the inhibitor by oxidation. A method was therefore devised for direct testing of the paper. The solution to be tested was put as a strip 2 cm. long on Whatman's 3MM paper, previously washed with 2N acetic acid, and distilled water, and the chromatogram was developed. The paper was dried (over an electric heater, not by a stream of air), examined under an ultra-violet lamp and fluorescent spots outlined, marked off with pencil lines into 10 segments, Rf 0–1·0, and put in large petri-dishes; nutrient agar was seeded with conidia of *N. crassa* and poured over the paper to give a thin film; surplus agar was trimmed away and the plates left for 18 hours. The surface of the agar was then painted with cotton blue in lacto-phenol and the plates examined under a binocular microscope illuminated from above. The young colonies could be easily observed and the region of inhibition marked. The rate of diffusion of the inhibitor in agar is slow and the zone of inhibition therefore coincides fairly nearly with the position on the paper of the inhibitor.

EXPERIMENTAL RESULTS

Fluorescent materials in oat roots

Oat seedling roots when viewed by ultra-violet light show bright blue fluorescence. The fluorescent materials were examined and described by Goodwin (1948, 1954) as the coumarin derivative scopoletin occurring together with its glucoside throughout the root except for the root-tip, and an unidentified glucoside which he called the root-tip glucoside or RTG occurring mainly in the root-tip. The inhibitor from the root described in the present work and Goodwin's RTG appear to be identical (Mitchell, H. K., and Maizel, J. V., 1960). When the roots are extracted by the method described above scopoletin is less readily adsorbed than RTG on to powdered paper but some is present in the eluate; it is adsorbed together with RTG on to charcoal from which it is eluted with 80 per cent. methanol, whereas it has not been found possible to elute RTG from charcoal. Scopoletin is not inhibitory to *O. graminis* or *N. crassa*.

Roots at various stages of early development were excised and squashed by a glass slide directly on to the base line of a 7-in.-deep sheet of Whatman's No. 1 paper which was run in n-propanol:1 per cent. ammonia 3:1. The results (Fig. 1) show the same distribution as that described by Goodwin (1954). Fluorescence is absent from the region of the crown upwards, none of the epicotyl being fluorescent. Visual examination under ultra-violet light of germinating excised embryos, and roots of growing seedlings, showed the meristems to be fluorescent from their inception, lateral roots being first

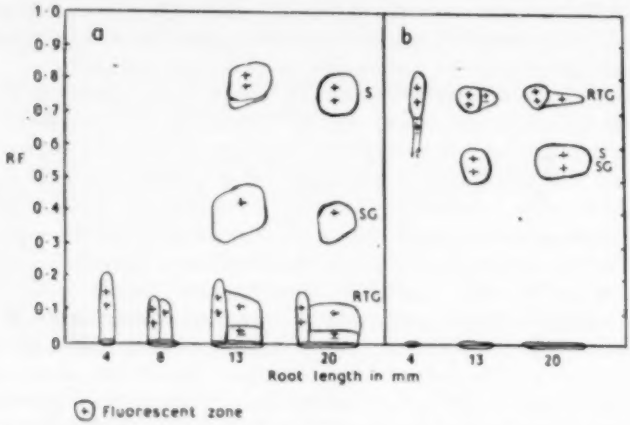


FIG. 1. Diagram showing position of fluorescent spots on a chromatogram when roots at different stages of early development are crushed directly on to the paper. The root-tip is on the left in each case.

Solvents (*a*) n-propanol: water 1:6.
(*b*) n-propanol: 1 per cent. ammonia 3:1.

S—scopoletin; SG—scopoletin glucoside; RTG—root-tip glucoside.

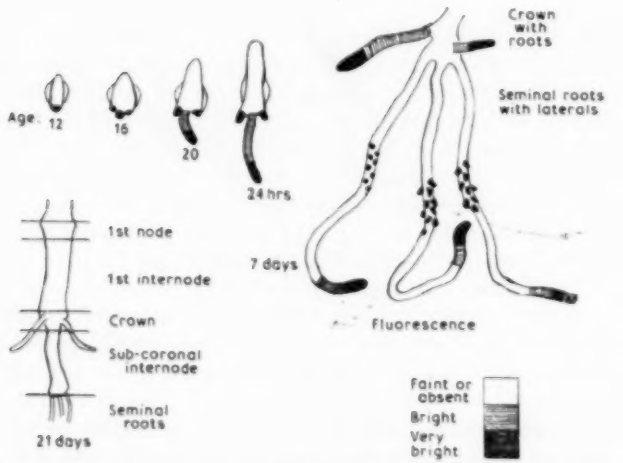


FIG. 2. Drawings of germinating oat embryos (excised) and of seedlings, showing structure and fluorescence. Not to scale.

visible as bright spots along the length of the seminal root. Secondary roots of older seedlings were also brightly fluorescent and the crown and sub-coronal internode slightly so (Fig. 2).

Distribution of inhibitor in roots and leaves

The zone of inhibition on chromatograms covered with agar seeded with *N. crassa* conidia coincided closely with the fluorescent spot due to RTG,

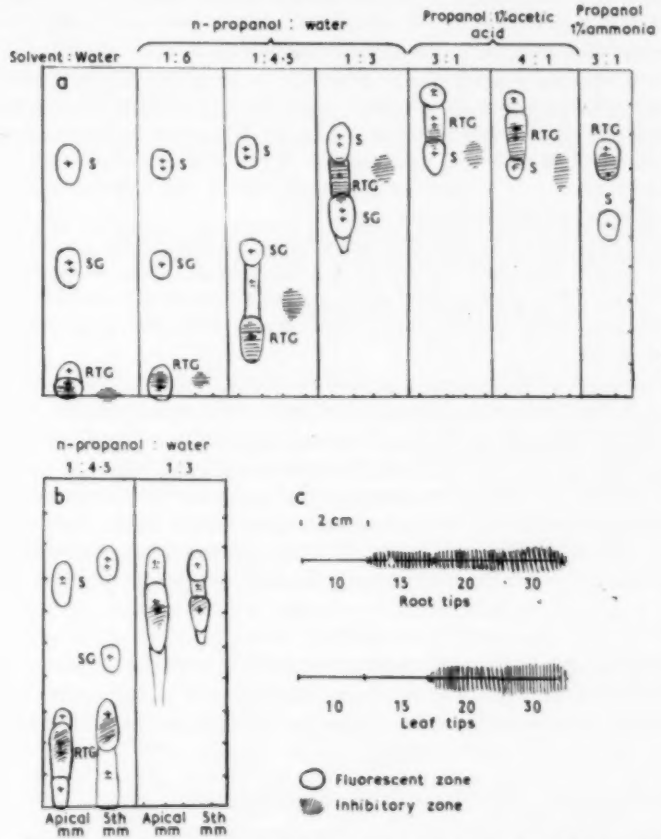


FIG. 3. Diagrams showing zones of fluorescence and of inhibition in chromatograms of

(*a*) extracts of oat roots and tops developed in different solvents;
(*b*) extracts of apical mm. and of 5th mm. segments of 3-day-old roots;
(*c*) extracts of 10, 15, 20, and 30 root- and leaf-tips applied over 2-cm. bars on paper but not developed.

(In (*a*) and (*b*) root extract is on the left and leaf extract on the right in each case.)

S—scopoletin; SG—scopoletin glucoside; RTG—root-tip glucoside.

although it usually occupied the lower half of this spot (Fig. 3). Although not fluorescent the inhibitor obtained from oat leaves appears closely related to that from roots in solubility, stability, activity, &c. This relation was examined

further by comparing chromatographic behaviour of the two extracts in different solvents. As described above, extracts of roots and leaves of comparable degrees of purity and activity were obtained in different ways and both were only crudely purified. The difference in molecular structure implied by the fluorescence of one of the compounds would also make identity of Rf values unlikely. However, Fig. 3 (*a*) shows that behaviour is comparable; both inhibitors remain on the base line when run in water and Rf values gradually increase with increasing proportions of propanol. Scopoletin and scopoletin glucoside are clearly separated from RTG by the more aqueous solvents; as the proportion of propanol increases the RTG runs ahead of scopoletin; in propanol: water, 3: 1, scopoletin and its glucoside run together with an Rf value of 0·54 and the RTG is at 0·74.

When these strips of paper are tested without developing, i.e. with crude extract on the base line, inhibition is proportional to the concentration of extract (Fig. 3 (*b*)); furthermore the near coincidence of the zone of inhibition with the area of the extract on the paper shows diffusion of the inhibitor in agar to be slow.

Distribution of fluorescent and non-fluorescent inhibitor in seedlings was examined further by assaying extracts from different segments. Seedlings were cut into appropriate 1-mm. segments by hand. In 2-day-old oat seedlings the first leaf is just about to break through the coleoptile; the latter can easily be removed by breaking at the base, the first leaf is then removed exposing the 'rudimentary leaves' just above the crown surmounting the unexpanded internodes of the stem. A 1-mm. segment was cut from the tips of these and also at the point just above the emergence of the seminal roots, the 'crown'. Table I

TABLE I

Diameter of colonies on 0·1-ml. streaks of agar relative to control (100 = 2·5 cm.) in dilutions of homogenates of 10 1-mm.-long segments of 2-day-old seedlings in 2 ml. water. Endosperm = milky contents of 3 germinated grains in 2 ml. water

Fungus	Segments /0·1 ml.	Endosperm	Roots			Leaves		Rudimentary leaves	Coleptile-tips	Crown
			Apices	Middles	Bases	Apices	Bases			
W	0·2	—	20	84	108	25	45	25	25	—
	0·1	—	25	104	126	50	81	30	51	—
	0·05	—	57	100	112	96	99	101	112	—
O_2	0·2	—	38	112	106	—	—	37	37	—
	0·1	—	61	106	101	—	—	54	70	—
	0·05	—	89	107	100	—	—	114	101	—
N	0·2	100	39	—	—	81	85	48	84	88
	0·1	109	50	—	—	94	98	81	96	115
	0·05	107	84	—	—	113	96	119	120	116

shows that the root apices yield most inhibitor and that the concentration falls off rapidly behind the tip; this agrees with the previous findings for RTG.

It can be seen that half of a 1-mm. segment of a root-tip in 1 ml. is sufficient to reduce growth of isolate W to 50 per cent. of the control; O_3 is less sensitive and *N. crassa* still less so. The rudimentary leaves provide the next highest yield followed by leaf-tips and coleoptile-tips, the ED_{50} for leaf-tips being 1 per ml.

The rapid fall in yield behind the root apex was confirmed by extracting the apical, 3rd and 5th mms. of 25 roots of 3-day-old seedlings in 0·5 ml. ethanol, transferring the filtered extract to water, and assaying at higher concentrations than in the previous experiment (Table II and Fig. 3 (*b*)).

TABLE II

Growth of W on assay slides relative to control (100) in dilutions of homogenates of 25 1-mm. segments of roots of 3-day-old seedlings in 0·5 ml. ethanol

	Root		
Segments/0·1 ml.	Apical mm.	3rd mm.	5th mm.
0·4	18	41	67
0·2	35	58	88

A further demonstration of yields from roots was obtained by placing whole roots from 2-day-old seedlings in petri-dishes and pouring seeded agar over them. After 18 hours the plates were examined; whole roots were surrounded by a clear zone of inhibition, equal in diameter along the length of younger roots, smaller behind the apex of older roots; neither endosperm nor hulls from the grain produce inhibition.

Older seedlings grown for 3 weeks in vermiculite in 10-in. pots watered with a culture solution were assayed for yield of different parts, but in this case results are hardly comparable quantitatively because of the difficulty of finding a basis of comparison between different organs. Fig. 2 shows the segments examined. Ten segments or organs of each type were grown in 3 ml. water and shaken with 1 g. powdered cellulose which was then eluted with 3 ml. 80 per cent. ethanol and the extract transferred to 1 ml. water; 0·3 ml. of each extract was put on a chromatogram paper and the remainder kept for assay. Fig. 4 shows distribution of fluorescent substances in the crown roots and of fluorescence and activity in the other segments, and Table III the assay results. They show that fluorescent or non-fluorescent inhibitor is present in all roots, nodes, and internodes examined. Scopoletin is also present throughout; like the RTG it is most abundant in the crown, the crown roots, and the old seminal roots. The crown contains the rudiments of new secondary roots as well as the stumps of old excised ones, hence its high content of fluorescent substances. Scopoletin is most concentrated towards the tip of the crown root and scopoletin glucoside is absent from the extracts of regions above the crown. The slight fluorescence of the extracts of the sub-coronal internode and of the first node is due to scopoletin only, and the inhibitor present is not fluorescent; a trace of RTG or fluorescent inhibitor seems to be present in the extracts from the first internode.

When older plants (12–14 weeks) grown in the field are examined the yield of inhibitor is very low. The ratio of meristem to mature tissue in roots and leaves is then very small and sap expressed from whole leaves or roots is barely inhibitory.

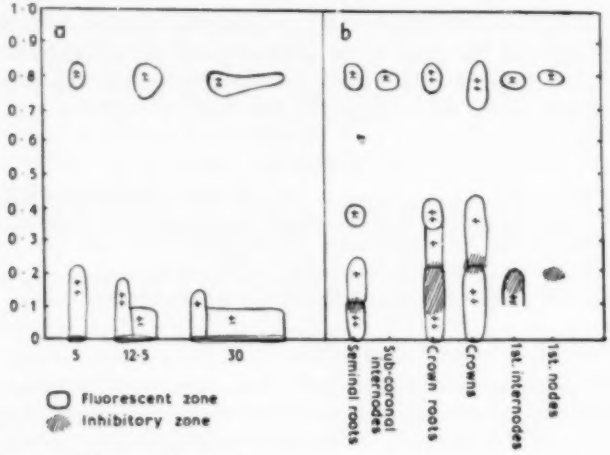


FIG. 4. Diagrams showing

(*a*) distribution of fluorescent substances in whole crown roots crushed directly on to paper;

(*b*) zones of fluorescence and of inhibition in chromatograms of extracts of different segments of 3-week-old seedlings.

Solvent, n-propanol : water, 1 : 6.

TABLE III

Diameter of W colonies relative to control in extracts of 10 organs or segments from 3-week-old seedlings. Extracts are not quantitatively comparable

Dilution	Old seminal roots	Sub-coronal internode	Crown roots	Crown	1st internode	1st node
1/6	19	19	19	19	19	19
1/20	27	28	19	19	29	19
1/50	38	57	68	68	90	27
1/100	67	—	96	90	—	62
Fluorescence	++	±	+++	++	+	±

Reaction of the fungi to crude sap and to semi-purified inhibitor

O. graminis var. *avenae* can grow in sap extracted from oat roots at dilutions at which the sap is still inhibitory to *O. graminis* itself. Yet the two varieties can hardly be differentiated in their reaction to the crudely purified inhibitor since although some O isolates have a higher threshold of inhibition, others have not (Turner, 1956, and Fig. 5).

It was suggested that when growing in the favourable medium of the cell

sap the O isolates could overcome the effects of the inhibitor (Turner, 1956). When equal quantities of root sap were put on two chromatography papers, one being developed and the other not, and both were tested by covering with *Neurospora* seeded agar, inhibition was found in each case, showing that, for *Neurospora*, the growth-stimulating substances in the sap do not mask the inhibitor. Assay by this method is unsatisfactory with *O. graminis* because

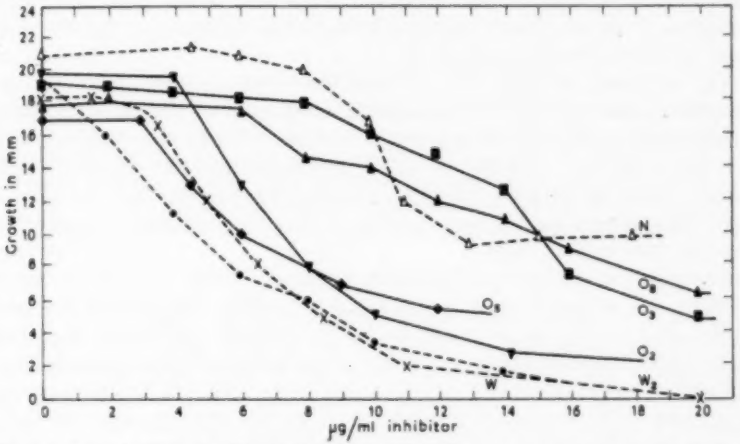


FIG. 5. Growth curves for W and O isolates and *N. crassa* on 0·1-ml. agar streaks containing various concentrations of dried inhibitor.

of the few spores produced, and assay of eluates is difficult because of instability of the inhibitor; however, by heavily loading the paper and eluting by immersing the strips in a small quantity of ethanol, inhibition of O_3 by the eluate from the segment Rf 0·7 (after development in propanol : 1 per cent. ammonia, 3 : 1) could be shown, whereas eluate from the undeveloped spot was not inhibitory, the inhibitor not being separated from growth stimulants, and the concentration being low.

A preliminary attempt was made to identify the fraction of the root extract active in masking the inhibitor for O_3, or used by O_3 in overcoming inhibitory effects, by testing eluates of different segments of chromatograms of root sap against semi-purified inhibitor, but results were not significant.

DISCUSSION

The leaf and root meristems of oats thus produce inhibitors, fluorescent in the root-tips and non-fluorescent in the leaf-tips. Yield from individual root-tips is the higher, extract from one root-tip in 2 ml. water effecting a 50 per cent. reduction in growth of isolate W whereas twice this concentration is required for leaf-tips. However, a higher yield (about $\times 4$) is obtainable from the tops of a tray of young seedlings than from the roots because of the larger number of leaf meristems. Concentration in the cells becomes less as the

tissues expand and mature; whether there is transport back from the apices is not known. The change at the region of the crown from fluorescent to non-fluorescent inhibitor is interesting, fluorescence due to RTG being absent from the sub-coronal internode but present again in the crown, because of adventitious root production; probably in the crown both forms of the inhibitor are present. The trace of RTG in the first internode may be due to the inclusion of some adventitious root rudiments. Apart from this the distribution of the two forms of the inhibitor thus depends on the meristems from which the tissue was derived.

The evidence indicates that the two inhibitors are essentially the same substance, distinguishable by presence or absence of fluorescence, which would thus depend on structure of a part of the molecule not concerned in its biological activity. The behaviour on chromatograms is sufficiently comparable, allowing for some difference in molecular structure and for different impurities present in the two extracts. The rate of diffusion in agar from paper is slow in both cases.

Although the inhibitor, in one of its forms, is present throughout the oat seedling, it is only in the roots and crown that it will be concerned in resistance of the plant to the root-infecting fungus *O. graminis*. This resistance, contrasted with susceptibility to var. *avenae*, is paralleled closely by the differential activity against the two fungi of expressed root sap, but much less closely by activity of the semi-purified inhibitor. That this capacity of var. *avenae* to overcome toxicity is due to a positive attribute, viz. production of an enzyme, rather than simply to growth stimulants in the sap, will be shown in a subsequent paper. Conditions for growth in the sap, however, apart from the presence of the inhibitor, are evidently favourable, and at appropriate dilutions of sap when the inhibitor is no longer active growth in sap is increased relative to the control.

ACKNOWLEDGEMENTS

I am indebted to the Agricultural Research Council for a Research Grant during the tenure of which this work was done; and to Mr. Cripps of the University Botany Department, Oxford, for help with devising the direct assay of inhibitory zones on chromatograms.

LITERATURE CITED

Goodwin, R. H., and Kavanagh, F. (1948). Fluorescing substances in roots. *Bull. Torrey bot. Cl.* **75**, 1.

—— and Pollock, B. M. (1954). Studies on roots: 1. Properties and distribution of fluorescent constituents in *Avena* roots. *Amer. J. Bot.* **41**, 516.

Mitchell, H. K., and Maizel, J. V. (1960). In the press.

Turner, E. M. (1940). The reaction of oats to different strains of *Ophiobolus graminis*. *Trans. Brit. mycol. Soc.*, **24**, 267.

—— (1952). The nature of the resistance of oats to the take-all fungus. *J. Exp. Bot.* **4**, 264.

—— (1956). Ditto, II. Inhibition of growth and respiration of *Ophiobolus graminis* and other fungi by a constituent of oat sap. Ibid. **7**, 80.

White, N. H. (1942). The genetics of *Ophiobolus graminis* Sacc. *J. Coun. Sci. Industr. Res. Aust.* **15**, 118.

Cherelle Wilt of Cacao

II. WILT IN RELATION TO YIELD

A. D. McKELVIE

West African Cocoa Research Institute[1]

Received 18 May 1959

SUMMARY

Experiments were carried out to study whether cherelle wilt is responsible for loss of yield in cacao. It was found that increasing or decreasing flower setting had no effect on yield as the crop was unrelated to the number of cherelles formed. Preventing setting till after the April 'flush' had no effect on yield. Variations in wilt between and within selections were studied, and it was concluded that yield is dependent on the nutrient status of the tree and independent of fluctuations in cherelle wilt.

INTRODUCTION

In a previous paper, McKelvie (1956) showed that young cacao pods, or 'cherelles', are specially liable to wilt at two stages in their development. It was postulated that both types of wilt occur as a result of a lack of hormones normally produced by the endosperm, causing a decrease in the uptake of water and food materials. Nichols (1957) investigated the growth substances in cacao, concluding also that wilt is probably under hormonal control and that reduction of wilt by synthetic growth substances might be possible. He detected an acidic accelerator (cacao auxin I), a neutral accelerator (cacao auxin II), and an acidic inhibitor. The production of auxin I coincided with the decrease in wilt between the first and second periods of wilt as described by the present author. Nichols was unable to correlate second wilt with hormone levels. He tentatively concluded that cherelle wilt may be due to interference with vascular transport or differentiation. From these results it seems likely that the hormone theory of wilt is correct. On this assumption, McKelvie (1957) carried out a detailed investigation into the effects of naphthalene acetic acid (NAA) on cherelles, even although earlier experiments had tended to disprove the findings of Naundorf (1950) and others that cherelle wilt could be greatly reduced by growth-substance sprays. McKelvie (1957) sprayed cacao trees with NAA at concentrations of 25, 100, and 250 p.p.m. and kept detailed records of wilt, but even pods 7 and 10 weeks old, and specially susceptible to wilt, failed to show any response to the NAA. It was concluded from these, and from earlier experiments by Murray (1952), that spraying with growth substances against cherelle wilt was unsuccessful. Even if applied chemicals could prove effective it should be remembered that at any one time there are flowers and pods of all sizes on the tree and that it would be impossible to time the sprays to affect only the required sizes of cherelle.

[1] Now at the Plant Breeding Institute, Cambridge.

The impracticability of controlling or reducing wilt by such means forces one to consider closely the question of how much yield is affected by cherelle wilt. If it seriously reduces yield then more efforts should be made to find a control. However, nobody has shown that wilt is a major factor in controlling yield and that a reduction in wilt would increase the crop. Havord *et al.* (1954) claimed that nitrogenous fertilizers reduced the percentage of cherelle wilt and increased yield. The reduction in wilt was small and most of the increased yield was due to an overall stimulation of the tree. Murray (1954) showed that the increase in yield from fertilizers was due to the production of more cherelles with no decrease in percentage wilt. It is important to ask, however, whether a tree, without additional fertilizers, is using its food reserves to the best advantage. The present experiments have been designed to investigate wilt in relation to yield and to decide whether attempts to reduce wilt are likely to be worth while.

EXPERIMENTAL METHODS

Most of the experiments consisted of detailed weekly recordings of flowers and pods on cacao trees. Since unpollinated flowers remain on the tree from 1 to 3 days depending on the environment, weekly counts of flowers have to be multiplied by a variable correction factor to convert them into total flowers opened each week. This correction factor was evaluated each week by observing 100 flowers and noting how long they remained on the tree if unpollinated.

Estimates of cherelles set each week (S) were calculated from the formula:

$$S = H_2 - H_1 + W_2,$$

where H_1 and H_2 are the numbers of healthy pods at weeks 1 and 2 respectively and W_2 is the number of wilted pods at week 2. No account was taken of cherelles which absciss within 7 days of setting as the number is negligible. Cherelles which are lost during this period probably fall because of insufficient pollination and do not wilt. All wilted cherelles were removed each week, since previous experiments had shown that this had no effect on yield.

Individual pre-treatment yield records for several years were available for all the trees used in the experiments. Subjective errors in recording flowers and pods were eliminated as much as possible by using the same recorders throughout the experiments and checking their results regularly.

RESULTS

Pollination and yield. Estimates of natural pollination in cacao have ranged from 0·5 to 20 per cent. of total flowers formed and it has often been suggested that yield is limited to a large extent by insufficient pollination. In January 1956, forty 9-year-old trees were selected for uniformity in appearance and from previous yield records. On 20 trees, in addition to insect pollinations, numerous hand-pollinations were carried out continuously from January to July, which is the duration of the flowering season, pollen being obtained randomly from neighbouring compatible trees. The other 20 trees were left as controls

to be pollinated by insects. Records were kept of all hand-pollinations and of the age at which the subsequent cherelles wilted. All ripe pods were individually weighed. Table I gives the results of this experiment.

TABLE I

Treatment totals	Hand-pollinated trees: Natural sets	Hand-pollinated trees: Sets from hand-pollinations	Hand-pollinated trees: Total	Control trees
Pods harvested 1956–7	324	169	493	536
Pods set	2,824	916	3,740	3,107
Pods wilted	2,500	747	3,247	2,571
Wet weight cocoa per pod (g.)	99	111	102	101
Pods harvested 1955–6 (pre-treatment)	—	—	304	328

'Pods harvested' includes all diseased and damaged pods past the age of wilting. The only significant differences between the two treatments are for pods set and pods wilted.

The additional hand-pollinations have not increased yield over the controls and there is almost a suggestion of a reduction. Pods from treated trees contained more cocoa when they were the result of hand-pollinations because most were low down on the main trunk where pods are always larger than on the branches. Table II shows that even on the poorer yielding trees in the experiment there was no disproportionate increase in yield from hand-pollinations compared with the control trees.

TABLE II

Pods per tree

	Hand-pollinated trees		Control trees	
	More than 30 pods per tree in 1955–6	Less than 30 pods per tree in 1955–6	More than 30 pods per tree in 1955–6	Less than 30 pods per tree in 1955–6
1956–7	60·5	24·3	66·6	28·7
1955–6 (pre-treatment)	44·7	20·1	36·8	24·2

Fig. 1 shows the number of pods which wilted each week throughout the year. On the treated trees there was very heavy wilt in April and June. By the end of June when the main crop is set there were equal numbers of cherelles on the two groups of trees. The crop on the treated trees was earlier because of hand-pollinations in April when there were few pollinating insects and the level of natural pollination was low.

Removal of sets. Because increased hand-pollinations did not increase yield it was decided to investigate whether a reduction in setting reduced yield. Seventy-two 8-year-old clonal trees were chosen and divided into 18 4-tree plots allowing 6 replications of the three treatments. The treatments were (A) removing $\frac{1}{3}$ of the newly formed sets each week, (B) removing $\frac{1}{5}$, and (C) control.

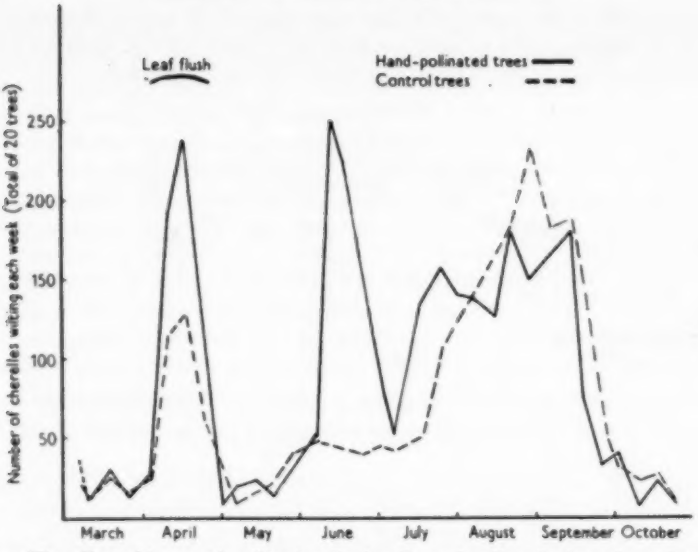


FIG. 1. The effect of increased pollination (by hand) on cherelle wilt throughout the year.

TABLE III

Totals per treatment

	½ removed A	½ removed B	Control C	Significance at 5% level
Pods harvested .	173	181	163	A = B = C
Pods wilted . .	605	846	1,077	C > B > A
Sets removed .	359	227	0	A > B > C
Total sets . .	1,137	1,254	1,240	A = B = C

TABLE IV

Treatment totals	Treated trees	Control trees: Before end of April	Control trees: After April	Total
Flowers formed. . .	21,524	15,416	28,900	44,316
Pods set	1,906	241	2,493	2,737
Pods wilted . . .	1,388	223	1,962	2,185
Pods harvested . . .	212	0	220	220
Wet weight cocoa pod (g.) .	105	0	114	114

Each week the trees were visited and the cherelles removed on the appropriate trees. Table III gives details of the number of pods harvested during the year.

There were many more cherelles on treatment C till August when heavy wilt reduced numbers to the level of A and B. Thus it appeared that regardless of previous thinning of young sets the trees reduced their crop to a common level as the pods for the main crop began to swell.

Prevention of flowering. It is well known that there is heavy wilting of young cherelles in March–April when there is a leaf flush, so that cherelles set from January to March are lost. In this experiment flowers and flower buds were removed from thirty-six 7-year-old clonal trees until the flush was finished, at the end of April, and then trees were allowed to set fruit. Thirty-six trees

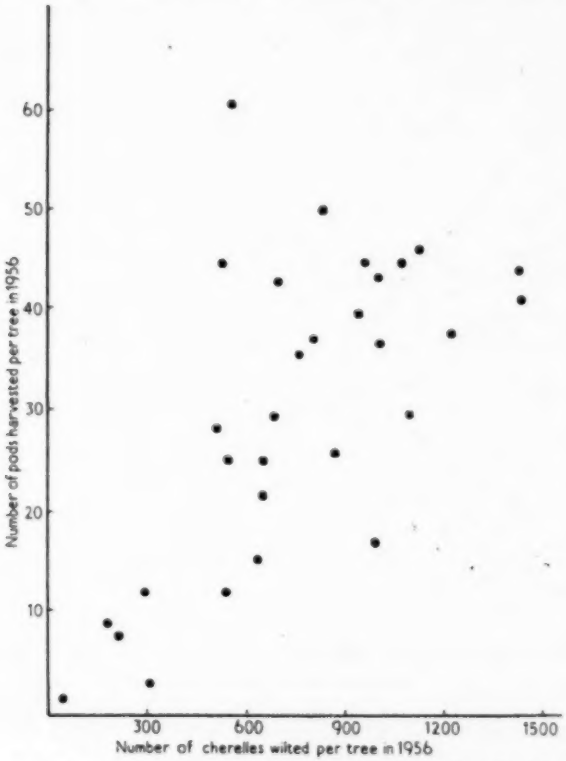


FIG. 2. [illegible]lation between pods harvested and cherelles wilted for 30 different selections growing in the same area. Averages for 5 trees of each selection.

were left as controls. Each week records were taken of wilting, and ripe pods were individually weighed. Table IV gives the results of this experiment. Yield differences for both total pods harvested and for weight of cocoa per pod were insignificant. Flowering remained low in the treated trees until July: consequently there were only half as many flowers on treated trees in May and June when the main crop set as on control trees. Setting was correspondingly low in May but yield was unaffected.

Variation of wilt between selections. Weekly counts of flowers, cherelles set and wilted, and pods harvested were made for 2 years on 150 trees of 30 selections

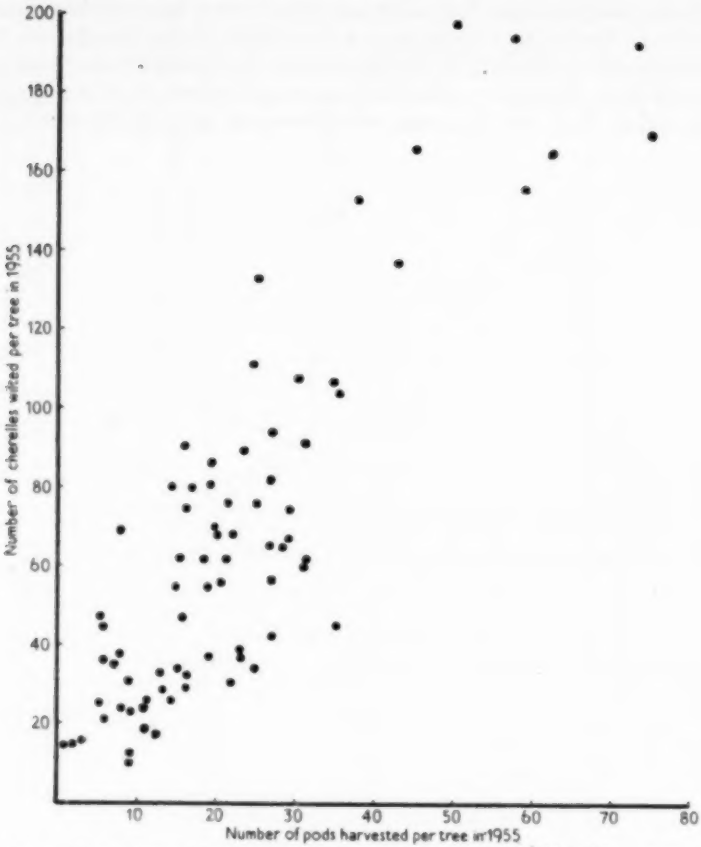


FIG. 3. The relation between pods harvested and cherelles wilted for clonal trees of selection ACU 85 in 1955.

to study how wilt is correlated with yield between selections. These trees included Amelonado, Trinitario, Criollo, and Amazon types. Fig. 2 shows the relation between selections, for wilt and yield for 1955–6. There is a poor correlation between number of wilted cherelles and yield, many varieties being high-yielding in spite of heavy wilt and others low-yielding in spite of a low level of wilt. There was an indication among the Amazon selections in this experiment that the amount of wilt depends on the parental cross and Hurd (1958) has found further evidence of this in the same experiment for 1957–8, and it is being investigated.

Variation of wilt within a selection. Ninety-six 10-year-old clonal trees of selection ACU 85 were closely studied from 1955 to 1957 in an attempt to correlate yield with wilting behaviour. Each week counts were made of

flowers, wilted cherelles, and ripe pods. Leaf counts and girth measurements were taken at yearly intervals.

Fig. 3 shows the relation for 1955 between the number of pods harvested and wilted cherelles for each tree. While the regression line is highly significant ($r = 0{\cdot}72$, D.F. $= 87$) there are many trees which show a poor fit and

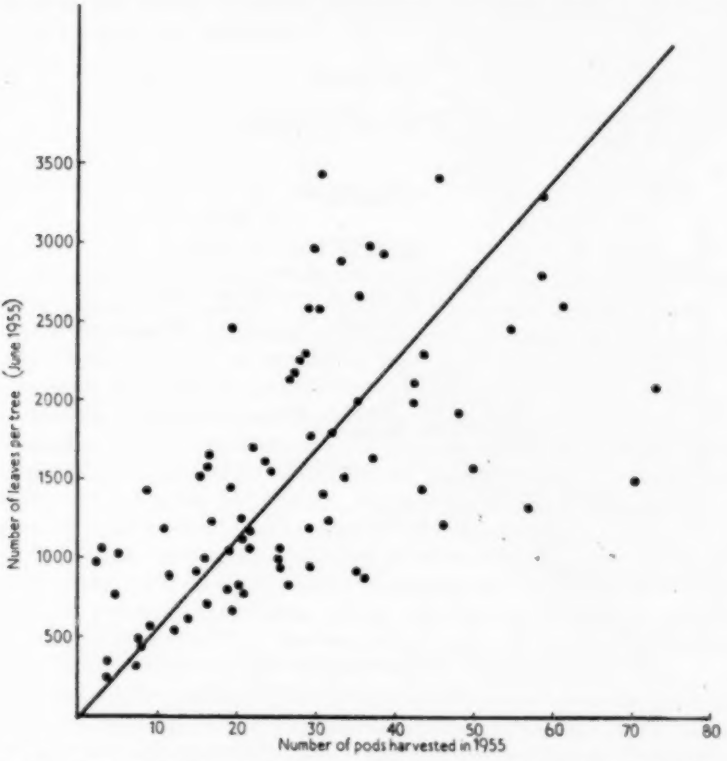


FIG. 4. The relation between pods harvested in 1955 and the number of leaves in June 1955 for selection ACU 85.

where many more cherelles wilted than would be expected. Each of these trees was visited but there was no apparent reason for any of the discrepancies. There were sufficient of these trees to make the problem important and the results were analysed closely in an attempt to elucidate it. All the records tended to show that the trees with most leaves had the most flowers, most wilted cherelles, and highest yield, but there were a number of exceptions as can be seen in Fig. 4, which shows yield for 1955 in relation to the number of leaves in June 1955. Several trees have many more or many less pods than would be expected from the number of leaves. Fig. 5 shows the yields for 1956

in relation to the regression line of Fig. 4 and it can be seen that in practically every case the yield for 1956 has tended to move towards this line. In other words it seems that those trees which produced more pods in 1955 than expected from their number of leaves, declined in yield in 1956, those trees with fewer pods than expected in 1955 increased in yield in 1956, while those trees on the regression line in 1955 tended to remain there in 1956.

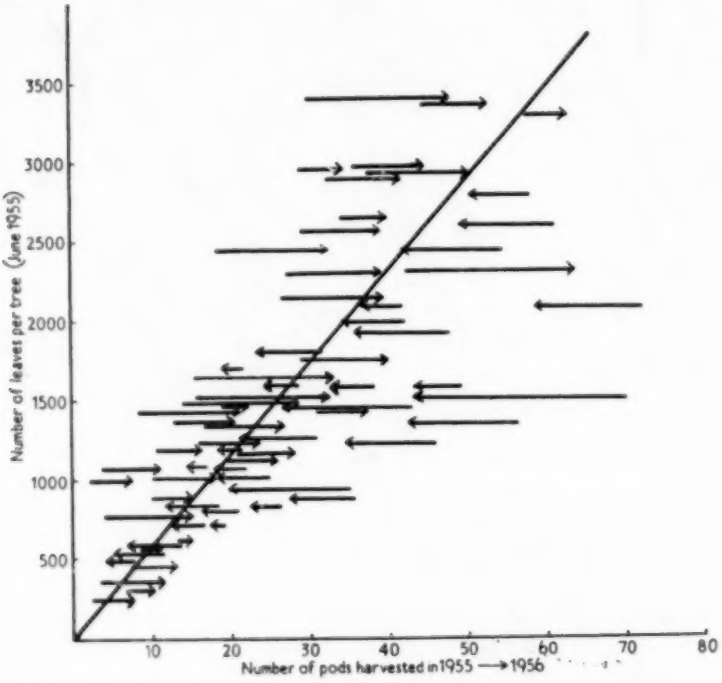


FIG. 5. Graph showing yield of selection ACU 85 in 1956 in relation to the regression line of Fig. 4. Arrows lead from yield in 1955 to yield in 1956. The tendency is for the yield to move towards the regression line.

Flush prevention. An experiment was carried out in 1955 on ninety-six 8-year-old clonal trees to study the effect of removing leaf buds, and thus preventing flushing, on wilt and yield.

Each week from February till September all leaf buds were pinched out as they began to open. Forty-eight trees were treated in this way and 48 left as controls. Weekly counts were taken as described in the previous experiments. Table V gives the results of this experiment. There were significantly more wilted cherelles and larger pods on the control trees but there was no significant difference for pods harvested. All yields were low, as small trees had to be chosen to facilitate bud removal. Where flushing was prevented very few of

the flowers which set in March–April wilted and an early crop was produced in August–September, whereas on control trees there was almost complete loss of cherelles during the April flush and the crop was not produced till October–November. By the end of September the treated trees had only 40 per cent. of the number of leaves on the control trees, so that carbohydrates must have been greatly lacking and thus it is not surprising that the weight of cocoa per pod was reduced.

TABLE V

	Cherelles wilted per tree	Pods harvested per tree	Wet weight cocoa per pod (g.)
Control trees . . .	39·2	14·3	101·6
Treated trees . . .	19·1	12·9	90·2

DISCUSSION

These experiments indicate that cherelle wilt is not normally the main physiological factor limiting the yield of cacao trees and that the number of ripe pods harvested is unaffected by the number of cherelles set earlier in the season. Under abnormal conditions of insufficient pollinating insects or of extreme drought these conclusions may not apply, but in general they seem to be valid for cacao growing at Tafo which is typical of West African conditions. Adams and McKelvie (1956) drew attention to the lack of knowledge of the optimum environment for cacao but even if such information is eventually obtained, for any given conditions there must be a limit to the amount of cacao a tree can produce and it appears that cherelle wilt is the mechanism by which the size of crop is regulated to the available food reserves. The experiments reported here show that neither increasing nor decreasing the number of cherelles, nor limiting flowering, affects yield but that as the crop is beginning to swell the incidence of cherelle wilt increases and the number of pods is reduced to a level consistent with the nutrient reserves of the tree.

This concept does not imply that wilt is the only factor limiting yield and that all the food materials of the tree are immediately available for the developing crop. The experiments with selection ACU 85 show that yield is closely correlated with the number of leaves and reveals the important point that if for any reason a tree in one season does not produce as large a crop as expected from the number of leaves it compensates the following year by increasing its crop in relation to the average of surrounding trees. The number of pods will be reduced by wilt in August to a level dictated by available nutrients but if thereafter food materials are released or manufactured in excess of crop requirements there will be a surplus either for the succeeding minor crop or for the next year's major crop. If on the other hand the tree has released to the crop all its reserves, at the expense of trunk growth for example, the tree will have overcropped and the next crop will be reduced.

Cherelle wilt between March and August can be caused by leaf flushes, by drought, by soil water-logging, and in general by most of the factors which are

known to induce fruit drop in other tree crops but as long as there are sufficient cherelles in August to produce a crop to utilize the available nutrients these earlier periods of wilt do not affect the size of the crop: they may, however, affect the time of harvest of the crop. Fig. 6 (adapted from data of Humphries, 1947) shows that the demand for nutrients by the crop early in the season is small in comparison with the demand in August as the crop begins to swell, and confirms the results of the present experiments that the August wilt is caused by lack of nutrients.

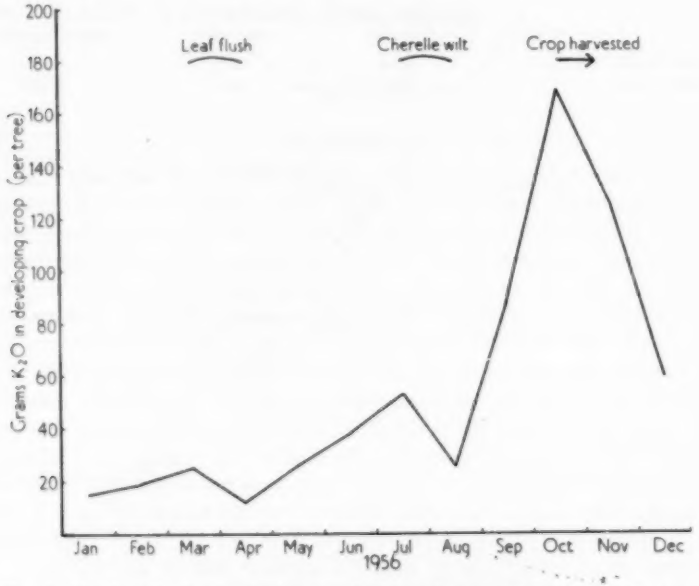


FIG. 6. Nutrients in the developing crop (expressed as gm. K_2O) during the year. Data from pod measurements in selection ACU 85 expressed in nutrient status from analytical data of Humphries (1947).

Addicott and Lynch (1955) summarized the theory of nutritional balance in plants, that the fruitfulness of many species is regulated by the relative abundance within the plant of carbohydrates and mineral nutrients (especially nitrogen) and that insufficient nutrients lead to embryo abortion, reducing the supply of auxin for the fruit and causing abscission. Eaton and Ergle (1953) postulated a 'relative fruitfulness' hypothesis for cotton: for each variety and environment the number of fruits per unit weight of leaves and stems is constant. They suggested that an anti-auxin produced by older fruit and auxin produced by the leaves interact in the fruiting branches, regulating abscission of younger fruits. Wadleigh (1944) suggested a simpler hypothesis: that a hormone from developing fruits stimulates abscission of younger fruits. The

amount of auxin produced in young fruits is less than in old fruits and thus the former would receive less nutrients, reducing their auxin production and causing abscission. Addicott and Lynch (1955) summarized these ideas on abscission and suggested that a high percentage of fruit abscission is caused by imbalance of carbohydrates and nitrogen but that ample supply of both leads to a constant percentage of fruit abscission with possible transient variations due to defruiting, auxin application, injury, drought, &c. This nutritional balance theory would seem applicable, on the basis of the present results, to cacao and explains the relatively constant level of cherelle wilt for any given variety and environment.

The natural reaction of most people working with cacao has been to think that cherelle wilt must be a factor limiting yield and that it should be capable of being reduced and giving higher yields. These present experiments have shown that this is unlikely and that the tree cannot support a larger crop with its available nutrients. With our present knowledge the only way to increase yield is by improved cultural conditions and the use of fertilizers. It is immaterial whether fertilizers increase yield by decreasing the percentage wilt or by increasing the number of flowers and cherelles with no change in wilt: the loss of nutrients to the tree through wilted cherelles is very small, and insufficient significantly to reduce the number of pods harvested.

Wharton (1958) has suggested that besides physiological wilt a number of cherelles can be lost through attack by *Phytophthora*. It is difficult in the field to distinguish between cherelles which are wilting and are consequently attacked by *Phytophthora* and cherelles primarily attacked by the fungus: this is particularly difficult because it is known that the growth-rate of wilting cherelles declines 1 week before yellowing symptoms appear. Wharton has also drawn attention to the abscission of cherelles within 1 week of setting. These young fruits can absciss for 1 week until the abscission layer becomes lignified. This phenomenon has been regarded by cacao breeders as due to incompatibility or failure to set properly and may be analogous with other fruits which fail to set properly owing to insufficient auxin. This abscission of recently set cherelles occurs most frequently at times when wilt is heavy. However, it seems clear that the loss of these young cherelles or the infection of some cherelles by *Phytophthora* will not influence yield in view of the conclusion already drawn. Any infection of cherelles past the age of wilting is very likely to reduce wilt.

It is concluded from these experiments that cherelle wilt is an unimportant factor in determining yield and that under normal conditions the yield is determined by the nutrient status and size of the tree regardless of how many cherelles are formed.

LITERATURE CITED

Adams, S. N., and McKelvie, A. D. (1956). Environmental requirements of cocoa in the Gold Coast. Cocoa Conference, London, 1955, 22.

Addicott, F. T., and Lynch, R. S. (1955). Physiology of abscission. *Ann. Rev. Plant Physiol.* **6**, 211.

EATON, F. M., and ERGLE, D. R. (1953). Relationship of seasonal trends in carbohydrate and nitrogen levels and effects of girdling and spraying with sucrose and urea to the nutritional interpretation of boll shedding in cotton. *Plant Physiol.* **28,** 503.

HAVORD, G., MALIPHANT, G. K., and COPE, F. W. (1954). The effect of fertilisers, shade and spacing on flowering, fruit-set and cherelle wilt. *A Report on Cacao Research*, I.C.T.A. Trinidad, 58.

HUMPHRIES, E. C. (1947). Wilt of cacao fruits (*Theobroma cacao*). IV. Seasonal variation in the carbohydrate reserves of the bark and wood of the cacao tree. *Ann. Bot.*, N.S. **11,** 219.

HURD, R. G. (1958). *Ann. Rept. West Afr. Cocoa Res. Inst.* 1957–8, 52.

MCKELVIE, A. D. (1956). Cherelle wilt of cacao. I. Pod development and its relation to wilt. *J. Exp. Bot.* **7,** 252.

—— (1957). *Ann. Rept. West Afr. Cocoa Res. Inst.* 1955–6, 32.

MURRAY, D. B. (1952). The effect of mineral injections and hormone sprays on flower setting and fruit development in cacao. *A Report on Cacao Research*, I.C.T.A. Trinidad, 1945–50, 22.

—— (1954). A shade and fertiliser experiment with cacao. Ibid. 1953, 52.

NAUNDORF, G., and VILLAMIL, G. (1950). Contribucion al estudio del la fisiologia del cacao. I. *Notas. Agronomicas*, **3,** 87.

NICHOLS, R. (1957). The growth substances of *Theobroma cacao*. *A Report on Cacao Research*, I.C.T.A. Trinidad, 1955–6, 33.

WADLEIGH, C. H. (1944). *Bull. Arh. agric. Exp. Sta.* No. **446**.

WHARTON, A. L. (1958). *Ann. Rept. West Afr. Cocoa Res. Inst.* 1957–8, 25.

continued from p. ii of cover]

Correspondence should be addressed as follows:

On editorial matters to Professor W. T. Williams, Botany Department, The University, Southampton.

With regard to membership of the Society for Experimental Biology, to the Hon. Botanical Secretary, Dr. G. E. Fogg, Department of Botany, University College, Gower Street, London, W.C. 1.

Members of the Society not yet receiving the Journal regularly who wish to become subscribers are asked to communicate with the Publications Secretary, c/o The Institute of Biology, 41 Queen's Gate, London, S.W. 7, who will deal with all matters of supply of the Society's journals to members.

On all other matters, to the publishers, Oxford University Press, Amen House, Warwick Square, London, E.C. 4.

EATON, F. M., and ERGLE, D. R. (1953). Relationship of seasonal trends in carbohydrate and nitrogen levels and effects of girdling and spraying with sucrose and urea to the nutritional interpretation of boll shedding in cotton. *Plant Physiol.* **28**, 503.

HAYSMS, G., MACPHANE, G. K., and COPE, F. W. (1954). The effect of fertilisers, shade and spacing on flowering, fruit-set and cherelle wilt. *4 Report on Cacao Research*, I.C.T.A. Trinidad, 28.

HEMBROUGH, E. C. (1947). Wilt of cacao fruits (*Theobroma cacao*). IV. Seasonal variation in the carbohydrate reserves of the bark and wood of the cacao tree. *Ann. Bot.*, N.S. **11**, 219.

HEUD, R. G. (1958). *Ann. Rept. West Afr. Cocoa Res. Inst.* 1957, 8, 52.

MCKELVIE, A. D. (1956). Cherelle wilt of cacao. I. Pod development and its relation to wilt. *J. Exp. Bot.* **7**, 252.

(1957). *Ann. Rept. West Afr. Cocoa Res. Inst.* 1955, 6, 32.

MURRAY, D. B. (1952). The effect of mineral injections and hormone sprays on flower setting and fruit development in cacao. *3 Report on Cacao Research*, I.C.T.A. Trinidad, 1945, 50, 22.

(1954). A shade and fertiliser experiment with cacao. Ibid. 1951, 52.

NAVASCUÉS, G., and VILLAMIL, G. (1950). Contribución al estudio del la fisiología del cacao. I. *Notas Agronómicas*, **3**, 87.

NICHOLS, R. (1957). The growth substances of *Theobroma cacao*. *3 Report on Cacao Research*, I.C.T.A. Trinidad, 1955, 6, 33.

WALLEGH, C. H. (1944). *Bull. Tech. agric. Exp. Stn.* No. **446**.

WHARTON, A. F. (1958). *Ann. Rept. West Afr. Cocoa Res. Inst.* 1957, 8, 25.

continued from p. ii of cover]

Correspondence should be addressed as follows:

On editorial matters to Professor W. T. Williams, Botany Department, The University, Southampton.

With regard to membership of the Society for Experimental Biology, to the Hon. Botanical Secretary, Dr. G. E. Fogg, Department of Botany, University College, Gower Street, London, W.C. 1.

Members of the Society not yet receiving the Journal regularly who wish to become subscribers are asked to communicate with the Publications Secretary, c/o The Institute of Biology, 41 Queen's Gate, London, S.W. 7, who will deal with all matters of supply of the Society's journals to members.

On all other matters, to the publishers, Oxford University Press, Amen House, Warwick Square, London, E.C. 4.

MICHROME STAINS

and Biological Reagents

Adenosine	Cytase	Methyl Green
Adenylic Acids	Fast Green	Ribonuclease
Carminic Acid	Fluormount	Sudan Black
Cedarwood Oil	Fluoroil	Toluidine Blue
Celloidin	Janus Green	Urease
Chlorazol Black	Lignin Pink	&c.

TRIFALGIC ACID, for cytology, electrophoresis, &c.

(Ref. *Irish J. Med. Res., April 1960, pages 182–6*)

HYMOUNT (refractive index 1·666)

AQUAMOUNT: synthetic, neutral, colourless aqueous mountant

62-page Catalogue available on request

ENCYCLOPAEDIA OF MICROSCOPIC STAINS
by Edward Gurr, 500 pages, Royal 8vo, 95s.

MICROSCOPIC STAINING TECHNIQUES, No. 4. (1st edit.) 66 pages. Price 6s. ($1.00 in U.S.A.) No. 3. (2nd edit.) 66 pages. Price 6s. ($1.00 in U.S.A.)

METHODS OF ANALYTICAL HISTOLOGY AND HISTOCHEMISTRY, by Edward Gurr. First edition, Royal 8vo, 334 pages. 70s. net.

EDWARD GURR, LTD.
42 Upper Richmond Road West
LONDON, S.W. 14
Telephones: PROspect 8051 and 7606

NEW BOOKS

A Large Stock of Recent Editions on BOTANY & ALLIED SCIENCES.
Catalogue sent on request.

SECONDHAND DEPT.
A select stock of recent editions available. Back numbers of scientific journals obtainable.

LENDING LIBRARY
Scientific and Technical
Annual Subscription £1. 17s. 6d.
Prospectus post free on request.
THE LIBRARY CATALOGUE revised to December 1956. To Subscribers, £1. 5s.; to Non-Subscribers, £2. 2s. Postage, 2s.

H. K. LEWIS & Co. Ltd.
136 GOWER STREET, W.C. 1
EUSton 4282

BOTANICAL BOOKS

New, Secondhand

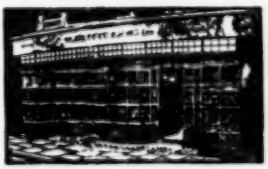
Old and Rare

WHELDON & WESLEY
LTD.
LYTTON LODGE
Codicote, Near Hitchin
Herts.

Telephone: CODICOTE 370

Libraries and small parcels of books purchased for cash

HEFFER'S



BOOKS ON BOTANY
IN ALL LANGUAGES

★

Catalogues available free; books and learned journals bought

★

W. HEFFER & SONS LTD.
Petty Cury · Cambridge

Printed in Great Britain at the University Press, Oxford, by Vivian Ridler, Printer to the University